REMARKS

Claims 1-43, 49-50, 54 and 56-63 stand cancelled. Claims 44, 46-48, 51-53 and 55 have been amended. No new matter has been added by virtue of these amendments; support therefore being found throughout the specification and in the original claims of the application.

As an initial matter, Applicant appreciates the courtesy extended by the Examiner as part of an informal, telephonic discussion with the undersigned Attorney on July 5, 2007. While no firm agreement was reached as to outstanding issues, among other things, prospective amendments to claims 53 and 55 were discussed with an eye toward gaining allowance of those claims.

Referring now to the Office Action, Applicant appreciates the indication of allowable subject matter, i.e. that the subject matter of claims 44, 45 and 60-63 are allowed. It is believed that the within amendments place all pending claims in condition for allowance. For instance, it is noted that the subject matter of allowable claim 60 has been incorporated in present (allowable) claim 44. Additionally, claim 53 has been amended to further define the features of the embodiment of the invention recited therein and documentary literature is offered in support of enablement for same.

Rejection under 35 USC §112, first paragraph

Claims 43, 46-48, 51-59 stand rejected under USC 35 §112, 1st paragraph, on the grounds of enablement. In particular, the position is taken that the specification is enabling for a method of treating asthma or COPD, but does not reasonably provide enablement for treating other diseases related to phosphodiesterase isoenzyeme.

Without acquiescing to the position taken, in order to expedite allowance of the application, claim 43 has been cancelled. Likewise, claims 46-48 and 51-59 have either been amended or cancelled in that regard.

With particular reference to claim 53, the subject matter thereof has been limited to "A method of treating a respiratory disorder... wherein the respiratory disorder is selected from the group consisting of allergic asthma, hay fever, allergic rhinitis, bronchitis, cystic fibrosis, and adult respiratory distress syndrome (ARDS)". Claim 55 has been amended to recite a particularly preferred embodiment thereof.

Additionally, in support of enablement for the methods recited in claims 53 and 55, Applicant offers five (5) publications attached hereto in Appendix A. It is respectfully submitted that such references establish the required nexus between the stated disorders and asthma, and their respective experimental models; thus amply satisfying the criteria for enablement. The references further demonstrate the efficacy of PDE4 inhibitors in the treatment of such diseases.

The review of Boswell-Smith *et al.* (*Br. J. Pharmacol.* 147:S252, 2006) provides a list of disease targets for different PDE isoenzymes. PDE3 is suggested to be useful for the treatment of airway disease and fertility. PDE4 is suggested to be useful for the treatment of allergic rhinitis, psoriasis, multiple sclerosis, depression, Alzheimer's disease, schizophrenia, memory loss, and cancer (see Table 2). The effects of PDE4 inhibitors in models of inflammation are discussed in the paragraph bridging pages S253 and 254 which is reproduced below:

> Indeed, the first generation PDE4 inhibitors were shown to be effective at inhibiting a wide range of inflammatory cell function *in vitro* including eosinophil (Dent *et al.,* 1991, lymphocyte (Giembycz *et al.,* 1996), basophil (Weston *et al.,* 1997) and neutrophil activation (Nielson et al., 1990). Furthermore, they were highly effective in suppressing inflammation in animal models of respiratory disease (Torphy & Undem, 1991). The ability of PDE4 inhibitors to also induce relaxation of isolated human bronchus (Cortijo *et al.,* 1993) gave rise to the hope that PDE 4 inhibitors could perhaps possess both anti-inflammatory and bronchodilator activity.

Inflammatory cells are mediators in the diseases listed, for example, in claim 53. Bronchial constriction is also a component of the pathology of the diseases listed including bronchitis and asthma. The review further discusses the efficacy of roflumilast (a PDE4 inhibitor) in the treatment of both asthma and COPD (column 1, page S254). These data demonstrate that it is not uncommon for a single agent to be effective in the treatment of multiple pulmonary inflammatory disorders that share pathologies.

The Lui *et al.*, (*J. Pharmacol. Expt. Therap.* **314**:846, 2005), *Schmidt et al.*, (*J. Allergy Clin. Immunol.* **108**:530, 2001), and Spond et al., (*Pulm. Pharmacol.Ther.* **14:** 157, 2001) also discuss the common pathologies of pulmonary inflammatory disorders, and that the effectiveness of an agent in the treatment of one disease can suggest the usefulness of the same agent for a related disease.

> For example, the abstract of Lui et al., states:
> The diseases of cystic fibrosis, chronic obstructive pulmonary disease (COPD), and chronic bronchitis are characterized by mucus-congested inflamed airways. Anti-inflammatory agents that can simultaneously restore or enhance muccociliary clearance through cystic fibrosis transmembrane conductance regulator activation (CFTR) may represent new therapeutics in their treatment.... Together with their potent anti-inflammatory properties, the potential for enhanced airway mucociliary clearance from CFTR activation may have contributed to the efficacy of PDE4 inhibitors in COPD and asthmatic patients. PDE4 inhibitors may represent new opportunities to combat cystic fibrosis and other respiratory diseases in the future.

In characterization of the diseases by pathology and looking at the specific pathologies and symptoms treated by a specific compound or class of compounds, the effectiveness of a drug for the treatment of a group of diseases, such as those provided in claim 53 or 55 can be expected.

Similarly, Schmidt *et al.*, teach that the common aspects of asthma and allergic rhinitis. The abstract states:

> The beneficial effects of phosphodiesterase 4 (PDE4) inhibitors in allergic asthma have been shown in previous preclinical and clinical studies. Because allergic rhinitis and asthma share several epidemiologic and pathophysiologic factors, PDE4 inhibitors might also be effective in allergic rhinitis.

In the Discussion section starting on page 534, the known results of PDE4 inhibitors are discussed as are the common elements of allergic asthma and allergic rhinitis (which can be seasonal *i.e.*, hay fever). These statements further demonstrate that the activity of a class of agents across multiple diseases with common pathologies, such as those in claim 53 or 55, can be predicted.

Spond *et al.*, discusses the commonalities between ARDS and COPD. Both diseases are characterized by the presence of pulmonary neutrophils and their extracellular deposition of granular products that are associated with an increase in airway dysfunction, mucus hypersecretion, tissue destruction and airway remodeling. These characteristics overlap with the characteristics of CF, COPD, and bronchitis as discussed by Lui *et al.* Spond *et al.*, teach the efficacy of PDE4 inhibitors in the treatment of acute lung injury which is a model for ARDS.

Miotla *et al.*, (*Am. J. Respir. Cell Mol. Biol.* **18:** 411, 1998) demonstrated that inhibition of PDE4 has three main anti-inflammatory effects, attenuation of tumor necrosis factor production, blockade of neutrophil sequestration in pulmonary capillaries, and inhibition of neutrophil activation (p. 418, col 2). Moreover, the PDE4 inhibitor was demonstrated to be beneficial inhibiting induction of lung injury even after neutrophil sequestration, suggesting that the class of compounds may be useful in treatment of ADRS. Applicant submits that having the three main anti-inflammatory

effects in lung listed, it would be expected that PDE4 inhibitors could be useful in the treatment of pulmonary inflammatory disorders characterized by the excess TNF production, and neutrophil sequestration and activation, such as asthma and COPD.

Applicant submits that by considering the substantial overlapping pathologies of asthma, allergic asthma, hay fever, allergic rhinitis, bronchitis, cystic fibrosis and adult respiratory distress syndrome (ARDS); the demonstration of the activities of the compounds of the invention in the specification; and the teachings of the art regarding the utility of one drug to treat multiple forms of pulmonary inflammation, that the specification is enabling for treating the pulmonary inflammatory disorders listed in claim 53 or 55.

Applicant respectfully submits that the within amendments obviate the rejection under 35 USC §112, first paragraph. Withdrawal of the rejection is therefore requested.

In view of the above amendments and remarks, Applicant believes the pending application is in condition for allowance.

Respectfully submitted,

Dated: September 4, 2007

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An **Appendix** follows - (Copies of 5 publications referred to above are included.)

APPENDIX A

British Journal of Pharmacology (2006) 147, S252–S257
www.nature.com/bjp

Phosphodiesterase inhibitors

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Phosphodiesterases are a diverse family of enzymes that hydrolyse cyclic nucleotides and thus play a key role in regulating intracellular levels of the second messengers cAMP and cGMP, and hence cell function. Theophylline and papaverine have historically been used therapeutically and are known to be weak inhibitors of PDE, but to what extent this contributed toward their clinical efficacy was poorly defined. However, the discovery of 11 isoenzyme families and our increased understanding of their function at the cell and molecular level provides an impetus for the development of isoenzyme selective inhibitors for the treatment of various diseases. This review focuses on the development of PDE3 inhibitors for congestive heart failure, PDE4 inhibitors for inflammatory airways disease and most successfully, PDE5 inhibitors for erectile dysfunction
British Journal of Pharmacology (2006) **147**, S252–S257. doi:10.1038/sj.bjp.0706495

Keywords: Phosphodiesterase; PDE4; PDE5; asthma; erectile dysfunction; inflammation

Abbreviations: CaM, calcium-calmodulin; cAMP, cyclic adenosine monophosphate; cGMP, cyclic guanosine monophosphate; EAR, early asthmatic response; LAR, late asthmatic response; LPS, lipopolysaccharide; NO, nitric oxide; PDE, phosphodiesterase; PGE, prostaglandin; TNF, tumour necrosis factor

Introduction

The phosphodiesterase (PDE) story begins with the work of Henry Hyde Salter in 1886. An asthmatic he noted that when he drank a strong cup of coffee on an empty stomach, his breathing eased, an effect attributed to the bronchodilator properties of caffeine. Although the mechanism of action at the time was unknown, it has since been shown that caffeine was acting as a non-selective, albeit weak, PDE inhibitor. Subsequently, analogues of caffeine including theophylline were successfully introduced as treatments for airway disease. The seminal work by Earl Sutherland and Ted Rall published in 1958, first identified the heat-stable nucleotide, cyclic adenosine monophosphate (cAMP) in liver extracts as a second messenger and suggested that it mediated many of the cellular effects of neurotransmitters and hormones. The discovery of cAMP was followed 5 years later by the identification of a second intracellular second messenger, cyclic guanosine monophosphate (cGMP), in rat urine (Ashman *et al.*, 1963). In this same study, PDE was identified as the enzyme capable of inactivating cAMP, and it was shown that this enzyme could be activated by magnesium ions and importantly could be inhibited by caffeine providing a plausible mechanism of action for the diverse activities of this drug (see Figure 1; Sutherland, 1958).

From a very early period, it was hypothesised that there were a number of different isoforms of PDE distinguished primarily by their substrate specificity and sensitivity to calcium-calmodulin (CaM) and these isoenzymes were numbered according to their elucidation order. They were first differentiated in the early 1970s in rat and bovine tissue (Beavo *et al.*, 1970). Initially, three enzymes were identified and known as CaM-PDE, cAMP-PDE and cGMP-PDE, which were further characterised by the use of selective inhibitors for these enzymes (Hidaka & Endo, 1984; Nicholson *et al.*, 1991). With the advent of the molecular age, the number of PDE isoforms identified increased and so in 1995, the nomenclature for the PDE family was standardised (Beavo, 1995). Today 11 isoenzyme groups, encompassing over 50 isoforms, have been identified including the recently characterised PDE4A11 (Wallace *et al.*, 2005) (see Table 1).

PDE activity is found in every cell in the body, although there is distinct cellular and subcellular distribution of the 11 isoenzymes, which has provided many possibilities for increasingly selective therapeutic targets (reviewed by Lugnier, 2005). In identifying isoenzyme selective targets for specific diseases, a substantial amount of work was undertaken by pharmacologists working in the U.K., particularly in characterising tissue expression, subcellular distribution and modulation of tissue function by isoenzyme selective inhibitors. This includes work by Nicholson and Shahid at Organon studying PDE expression in cardiac tissues and the airways (de Boer *et al.*, 1992; Torphy *et al.*, 1993; Shahid & Nicholson, 1995) and Miles Houslay and co-workers in Glasgow who have contributed significantly to our understanding of PDE4 and its various subtypes (Houslay, 2001).

The development of PDE3 inhibitors to treat congestive heart failure

PDE3 has high affinity for cAMP but can also hydrolyse cGMP. However, it hydrolyses cAMP at 10 times the rate it hydrolyses cGMP and therefore cGMP effectively acts as a competitive inhibitor for cAMP and consequently for PDE3 (Lugnier, 2005). As a result of its high expression in both the vasculature and the airways, PDE3 was identified as a potential therapeutic target in cardiovascular disease and

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asthma, and indeed, PDE3 inhibitors have subsequently been shown to relax vascular and airway smooth muscle, inhibit platelet aggregation (reviewed by Barnes *et al.*, 1988) and induce lipolysis (Manganiello *et al.*, 1995). However, the unequivocal effect of PDE3 inhibitors as positive inotropic agents provided a strong rationale for developing such drugs for the treatment of chronic heart disease (Nicholson *et al.*, 1991). A number of PDE3 selective inhibitors, including milrinone, were developed to treat patients with heart failure. However, chronic treatment with milrinone was associated with an increased risk of mortality and has consequently somewhat jaundiced the view of PDE3 as a drug target (Packer *et al.*, 1991). Nonetheless, milrinone is still used in the acute treatment of heart failure, and cilostazol, another PDE3 inhibitor, is used in the treatment of intermittent claudication.

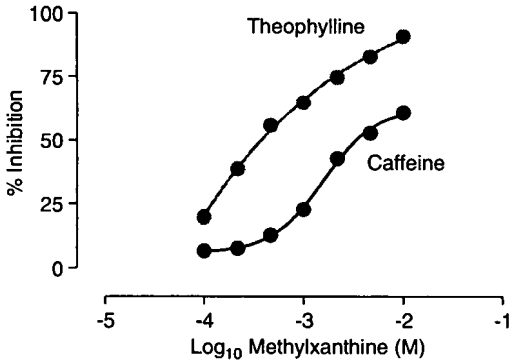

Figure 1 Inhibition of cAMP-PDE activity by methylxanthines. cAMP was incubated with purified cAMP-PDE at 30°C for 30 min in the presence of increasing concentrations of methylxanthines. Data redrawn from Butcher & Sutherland (1962).

Development of PDE4 inhibitors for the treatment of inflammatory airways disease

PDE4, formerly known as cAMP-PDE, is a cAMP-specific PDE and is the predominant isoenzyme in the majority of inflammatory cells, with the exception of platelets, implicated in inflammatory airways disease. It is expressed in the airways smooth muscle, brain and cardiovascular tissues (Muller *et al.*, 1996) and is the largest PDE subfamily with over 35 different isoforms identified thus far. The molecular structure, compartmentalisation and function have been extensively investigated (Houslay, 2001) and as such, PDE4 is the most widely characterised PDE isoenzyme. In the early 1970s, rolipram, a cAMP-PDE inhibitor, was developed as a potential drug to treat depression as it was demonstrated that elevation of cAMP could enhance noradrenergic neurotransmission in the central nervous system. Although rolipram proved to be an effective antidepressant, side effects of nausea and gastrointestinal disturbance terminated its clinical development (Scott *et al.*, 1991). In addition, the worldwide success of serotonin selective reuptake inhibitors in treating depression usurped PDE4 inhibitors as a potential therapy in this field. Nonetheless, there is a current resurgence underway in this area (Renau, 2004) and in addition, to the development of PDE4 inhibitors to treat other CNS indications such as memory enhancement (Inflazyme pharmaceuticals, 2005).

The success of theophylline in treating asthmatic patients (see also Barnes, this issue), the finding that raising intracellular levels of cAMP within inflammatory cells inhibited their function and the wide distribution of PDE4 in inflammatory cells and the lung led to the exploration of isoenzyme selective PDE4 inhibitors as potential treatments for airway disease (Torphy & Undem, 1991). Indeed, the first generation PDE4 inhibitors were shown to be effective at inhibiting a wide range of inflammatory cell function *in vitro* including eosinophil

Table 1 The PDE superfamily

PDE isoenzyme	*No. of isoforms*	*Substrate*	*Km* (μM) *cAMP*	*Km* (μM) *GMP*	*Tissue expression*	*Specific inhibitors*
1	8	Ca^{2+}/calmodulin-stimulated	1–30	3	Heart, brain, lung, smooth muscle	KS-505a
2		cGMP-stimulated	50	50	Adrenal gland, heart, lung, liver, platelets	EHNA (MEP-1)
3	4	cGMP-inhibited, cAMP-selective	0.2	0.3	Heart, lung, liver, platelets, adipose tissue, inflammatory cells	Cilostamide Enoxamone Milrinone Siguazodan
4	20	cAMP-specific	4		Sertoli cells, kidney, brain, liver, lung, inflammatory cells	Rolipram, Roflumilast Cilomilast
5	3	cGMP-specific	150	1	Lung, platelets, vascular smooth muscle	Sildenafil, Zaprinast
6		cGMP-specific		60	Photoreceptor	Dipyridamole
7	3	cAMP-specific, high-affinity	0.2		Skeletal muscle, heart, kidney, brain, pancreas, T lymphocytes	BRL-50481
8		cAMP-selective,	0.06		Testes, eye, liver, skeletal muscle, heart, kidney, ovary, brain, T lymphocytes	none
9	4	cGMP-specific,		0.17	Kidney, liver, lung, brain	BAY 73-6691
10	2	cGMP-sensitive, cAMP-selective	0.05	3.0	Testes, brain	none
11	4	cGMP-sensitive, dual specificity	0.7	0.6	Skeletal muscle, prostate, kidney, liver, pituitary and salivary glands, testes	none

(Dent *et al.*, 1991), lymphocyte (Giembycz *et al.*, 1996), basophil (Weston *et al.*, 1997) and neutrophil activation (Nielson *et al.*, 1990). Furthermore, they were highly effective at suppressing inflammation in animal models of respiratory disease (Torphy & Undem, 1991). The ability of PDE4 inhibitors to also induce relaxation of isolated human bronchus (Cortijo *et al.*, 1993) gave rise to the hope that PDE4 inhibitors could perhaps possess both anti-inflammatory and bronchodilator activity.

A number of pharmaceutical companies went on to develop potent second generation PDE4 selective inhibitors, and scientists at Celltech in Slough U.K., developed CDP840 (Hughes *et al.*, 1996), which in 1997, became the first orally active PDE4 inhibitor to demonstrate a beneficial effect in patients with asthma at doses producing no reported serious adverse effects. CDP840 had no direct bronchodilator activity nor did it inhibit acute bronchoconstriction in response to antigen challenge. This acute response is referred to as the early asthmatic response (EAR) and is known to be a consequence of mast cell degranulation and release of mediators including histamine as a result of antigen binding to high affinity IgE cell surface receptors. However, CDP840 did significantly suppress the late asthmatic response (LAR) by 30% at doses that did not elicit significant gastrointestinal side effects (Harbinson *et al.*, 1997). This was a very significant observation, as the LAR is viewed by clinicians to represent the inflammatory component of airway disease and showed that PDE4 inhibitors could indeed be anti-inflammatory drugs and that it was possible to obtain this effect without the side effects that dogged earlier drugs of this class.

Cilomilast developed by Ted Torphy and co-workers at SmithKline Beecham (now GSK) and roflumilast developed by Dr C Schudt and co-workers at Byk Gulden (now Altana) are orally active selective PDE4 inhibitors in late clinical development (Brown, 2005; Rabe *et al.*, 2005). In COPD patients, cilomilast significantly improved FEV_1 and quality of life scores, as well as reducing exacerbation rates, although it has still to be approved by the FDA. However, gastrointestinal disturbances such as emesis and nausea are still evident with this drug such that it is likely that the doses currently used in man are at the bottom end of the dose response curve and therefore the optimal effects of this class of drug are not being achieved with cilomilast. Roflumilast (250 and 500 μg, p.o.) significantly suppressed LAR by 27 and 43%, respectively, in patients with asthma. It also reduced the EAR (25 and 28%, respectively), although by a smaller margin than the LAR (Van Schalkwyk *et al.*, 2005). Significantly, studies have demonstrated the efficacy of roflumilast in patients with both asthma and COPD, where roflumilast improved lung function and reduced exacerbation rates, and thus it remains a promising new therapy to treat this disease (Rabe *et al.*, 2005). One recent study has shown that roflumilast is equivalent to taking inhaled beclomethasone diproprionate in the treatment of mild to moderate asthma, suggesting that such drugs may prove to be a viable alternative therapy to inhaled glucocorticosteroids (reviewed by Lipworth, 2005). Roflumilast is undergoing further clinical evaluation in patients with both asthma and COPD, although it is still a drug that has significant gastrointestinal side effects, particularly at high doses, which may prove problematic.

Thus, there still remains a challenge to design even better PDE4 inhibitors with an improved therapeutic index and a number of different strategies are being pursued to achieve this. The work of Souness & Rao (1997) working at Rhone Poulenc Rorer, latterly Aventis, in Dagenham suggested that PDE4 existed in two distinct conformations, one present predominantly in the CNS and parietal glands, which binds rolipram with high affinity termed HPDE4, and one, which binds rolipram with low affinity that is mainly present in inflammatory cells termed LPDE4. Accordingly, binding to the HPDE4 was predicted to be related to the adverse side effects associated with rolipram. However, cilomilast has reduced potency against HPDE4 but nonetheless is still emetic in patients treated with this drug. Another approach followed the recognition that there were four genetically distinct PDE4 subtypes; termed PDE4A-D. Conti and co-workers were the first to produce PDE4 knock-out mice deficient in either PDE4B or PDE4D and studies with these mice suggested that PDE4D was associated with emesis (Robichaud *et al.*, 2002), and in the development of airways hyperresponsiveness in response to cholinergic stimulation (Hansen *et al.*, 2000). Further studies with PDE4B knock-out mice showed that this PDE4 subtype was essential for LPS-induced generation of the cytokine TNF-α and thus, a PDE4B selective inhibitor could potentially be an effective anti-inflammatory agent without inducing emesis (Jin & Conti, 2002). Another potential avenue that could be exploited to improve drug selectivity and reduce side effects would be the targeting of specific PDE4 isoenzymes that are only expressed under inflammatory situations (Chan *et al.*, 2003). To date there is no evidence for altered PDE4 expression and function in inflammatory cells from asthmatic subjects (Landells *et al.*, 2001; Jones *et al.*, 2005) although increased expression of PDE4A4 has been documented in macrophages from subjects with COPD (Barber *et al.*, 2004). Whether selective targeting of this enzyme will lead to a better drug than roflumilast remains to be seen. In addition, the expression of PDE7 in inflammatory cells has been acknowledged and while inhibition of this enzyme alone does not suppress inflammatory cell function, however, combined use of PDE4 with PDE7 inhibitors provides a greater inhibition than PDE4 alone. Therefore, a hybrid PDE4/7 inhibitor may provide more effective anti-inflammatory activity and reduce side effects. It is of interest, therefore, that novel PDE4 inhibitors are now being developed, which claim to lack significant gastrointestinal side effects, including HT0712 (Inflazyme pharmaceuticals, 2005) and GRC 3886 (Glenmark Pharmaceuticals, 2005).

PDE5 inhibitors for the treatment of erectile dysfunction

A variety of treatments have been historically used to treat erectile dysfunction that influence the cyclic nucleotide signalling pathway in vascular smooth muscle including PGE_1, papaverine and pentoxifylline. It was recognised that papaverine and pentoxifylline mediated vasorelaxation by a number of mechanisms including non-selective PDE inhibition (Allenby *et al.*, 1991) and these drugs can be considered as forerunners to the clinically successful PDE5 inhibitors used today for the treatment of erectile dysfunction, although at the time, PDE5 inhibition as a mechanism to account for the actions of these particular drugs was not established.

PDE5, a cGMP-specific PDE was first identified in rat platelets in 1978 and was originally known as cGMP-PDE (Hamet & Coquil, 1978). Early on it was shown that cGMP-

PDE could be specifically inhibited by zaprinast, and this was widely used to explore the functional role of what we now know as the PDE5 isoenzyme (reviewed by Murray, 1993). Zaprinast was designated M&B22948 by May and Baker in Dagenham and designed as a mast cell stabilising drug for treating allergic diseases, and in this capacity M&B22948 became the first orally active isoenzyme selective PDE5 inhibitor to be given in man. It was originally administered to patients with exercise-induced asthma and was shown to have moderate bronchodilator effects (Rudd *et al.*, 1983). However, PDE5 inhibitors had no inhibitory effect on inflammatory cells other than mast cells, but they were able to induce vascular smooth muscle relaxation and therefore PDE5 was considered a possible therapeutic target in cardiovascular disease (Murray, 1993). Indeed, it was demonstrated that an elevation in cGMP mediated by zaprinast was associated with vascular smooth muscle relaxation of isolated rat aorta (Lugnier *et al.*, 1983; Rapoport & Murad, 1983); observations which led to the initiation of research programmes by a number of pharmaceutical companies to develop PDE5 inhibitors for a range of diseases. Pfizer in Sandwich, Kent, in particular, took the PDE5 inhibitor sildenafil into the clinic as a treatment for angina pectoris.

Other laboratories were undertaking studies to enhance understanding of the vascular and neurological control of the corpus cavernosum to better determine the basis of erections. In 1990, it was reported that electrical field stimulation (EFS) induced relaxation of rabbit corpus cavernosum smooth muscle cells. This response was also correlated with the formation of nitric oxide (NO) and a rise of intracellular levels of cGMP within smooth muscle cells, suggesting that enhanced NO production could potentially mediate penile erection (Ignarro *et al.*, 1990). This was followed by the seminal work of Rajfer *et al.* (1992) demonstrating that the NO pathway was also triggered by EFS stimulation in human corpus cavernosum to bring about relaxation, a mechanism which was reduced in tissue obtained from patients with impotence. As part of a very thorough investigation of this NO pathway regulating the relaxation of the corpus cavernosum, it was demonstrated that the PDE5 inhibitor zaprinast could enhance NO-induced relaxation of isolated corpus cavernosum (Rajfer *et al.*, 1992). These findings suggested that PDE5 inhibitors may also be useful for treating impotence (reviewed by Murray, 1993) (see Figure 2).

The data from the Phase 1 clinical trials with sildenafil in angina patients proved disappointing. However, while sildenafil provided no significant therapeutic improvement over the existing nitrate therapy, it was noted that one of the commonly reported side effects in this study was penile erection. This observation led to the important decision by Pfizer to change the focus of the sildenafil research programme to investigate their drug as a potential treatment of erectile dysfunction. In late 1993, the first clinical trial examining the efficacy of sildenafil for the treatment of erectile dysfunction was undertaken and it confirmed the potential of the drug to treat patients with this condition (Boolell *et al.*, 1996). Over the next $4\frac{1}{2}$ years, over 5000 patients received sildenafil in clinical trials and in March 1998, the FDA approved it for the treatment of erectile dysfunction. Since then, an estimated 177 million prescriptions in over 120 countries have been written and sildenafil has had a revolutionary impact on the understanding and treatment of this common condition. *In vitro* studies with

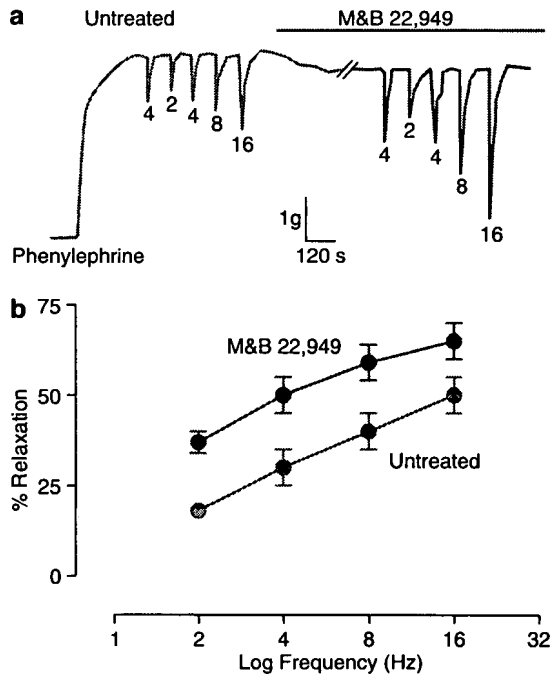

Figure 2 The PDE5 inhibitor M&B 22,949 (Zaprinast) augmented relaxation of human corpus cavernosum to electrical field stimulation (Hz). Data showing relaxation of human corpus cavernosum in an isolated preparation (a) or the mean±s.e.m. for 11 subjects (b); to different stimulation frequencies in untreated and M&B 22,949 (1–3 mM)-treated tissue. Data re-drawn from Raijfer *et al.* (1992).

Table 2 Disease targets for isoenzyme selective PDE inhibitors

PDE family	*Disease targets*
2	Sepsis, Acute Respiratory Distress Syndrome (ARDS)
3	Airways disease, fertility
4	Allergic rhinitis, psoriasis, multiple sclerosis, depression, Alzheimer's disease, schizophrenia, memory loss, cancer, dermatitis
5	Pulmonary hypertension, female sexual dysfunction, cardiovascular disease, premature ejaculation, stroke, leukaemia, renal failure
7	Inflammation

isolated human corpus cavernosum, similar to the earlier studies by Rajfer *et al.* (1992), were undertaken and these showed that sildenafil is approximately 240 times more potent than zaprinast at inhibiting PDE5 (Ballard *et al.*, 1998). Nonetheless, sildenafil is sometimes associated with visual disturbances due to activity against PDE6, an enzyme found in the retina, and also has a relatively short half-life. Therefore, while sildenafil has been very successful, it has some limitations and this has led to the development of newer PDE5 inhibitors. Two more PDE5 inhibitors, vardenafil and tadalafil, are now approved for use as treatments for erectile dysfunction. Vardenafil is more potent than sildenafil and tadalafil, and has a half-life of approximately 17 h, which allows more natural engagement of sexual activity. Furthermore, tadalafil is far less active against the PDE6 isoenzyme (selectivity ratio vs PDE5: 780) than either sildenafil (6.8) or

vardenafil (2.9) and consequently, the incidence of visual side effects associated with PDE6 inhibition in the photoreceptor cells is greatly reduced (<0.1% tadalafil compared with 3% sildenafil) (Maggi *et al.*, 2000).

Conclusion

Non-selective PDE inhibitors including theophylline and papaverine have been used therapeutically for over 70 years for a range of diseases. However, it is only in the last 10 years, that potent PDE selective drugs have begun to make an impact in the treatment of disease, and the worldwide success of sildenafil in treating erectile dysfunction is evidence of the effect such drugs can have. Selective PDE inhibitors are being investigated in a wide range of diseases (summarised in Table 2) including the use of PDE2 inhibitors in sepsis; PDE5 inhibitors to treat sexual dysfunction in females, cardiovascular disease and pulmonary hypertension; and PDE4 inhibitors to treat asthma, COPD, allergic rhinitis, psoriasis, multiple sclerosis, depression, Alzheimer's disease and schizophrenia. As we increase our understanding of the physiological roles of the individual PDE isoforms, in parallel with the development of even more selective inhibitors of these enzymes, it is highly likely that better therapeutically active drugs will emerge.

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The phosphodiesterase 4 inhibitor roflumilast is effective in the treatment of allergic rhinitis

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Background: The beneficial effects of phosphodiesterase 4 (PDE4) inhibitors in allergic asthma have been shown in previous preclinical and clinical studies. Because allergic rhinitis and asthma share several epidemiologic and pathophysiologic factors, PDE4 inhibitors might also be effective in allergic rhinitis.

Objective: The main objective of this study was to investigate the efficacy of oral roflumilast (500 μg/day) in allergic rhinitis.

Methods: In a randomized, placebo-controlled, double-blinded, crossover study, 25 subjects (16 male, 9 female; median age, 28 years) with histories of allergic rhinitis but asymptomatic at screening received roflumilast (500 μg once daily) and placebo for 9 days each with a washout period of at least 14 days in between treatment periods. In each of the treatment periods, controlled intranasal allergen provocation with pollen extracts was performed daily beginning the third day of treatment, each time approximately 2 hours after study drug administration. Five and 30 minutes after each allergen provocation, rhinal airflow was measured by means of anterior rhinomanometry and the subjective symptoms obstruction, itching, and rhinorrhea were assessed by means of a standardized visual analog scale.

Results: Rhinal airflow improved almost consistently during the 9 days of roflumilast treatment, and it was significantly higher at study day 9 on roflumilast in comparison with placebo, a result also found for itching and rhinorrhea. With respect to the subjective obstruction score, a significant difference in comparison with placebo could be demonstrated within 4 days.

Conclusion: This study shows that a PDE4 inhibitor, roflumilast, effectively controls symptoms of allergic rhinitis. Thus PDE4 inhibitors might be a future treatment option not only in allergic asthma but also in allergic rhinitis or the combination of the 2 diseases. (J Allergy Clin Immunol 2001;108:530-6.)

Key words: Allergic rhinitis, PDE4 inhibitors, asthma, rhinomanometry

Abbreviation used
PDE: Phosphodiesterase

Allergic rhinitis is a common disease that is estimated to affect approximately 8% to 24% of the population in industrialized countries.[1,2] Although the disease is generally viewed as a minor nuisance, its symptoms—nasal obstruction, itching, rhinorrhea, and concomitant alteration of the eyes—generally impair the health-related quality of life in patients with either the perennial form or the seasonal form. Whereas the latter is induced mainly by natural pollen exposure, the former is triggered by various environmental allergens (eg, house dust and animal hair). On immunologic exposure, mediators such as prostaglandin D2, leukotriene E4, tryptase, and histamine are generated, reflecting the early allergic reaction. The subsequent allergic inflammation is mostly due to recruitment of eosinophils and metachromatic cells.[3]

The clinical impact of allergic rhinitis is enhanced by associated complications, such as sinusitis due to nasal obstruction, and by the rhinitis-asthma link; up to 40% of children and adolescents with allergic rhinitis also develop allergic asthma.[4,5]

If complete allergen avoidance cannot be achieved or if immunotherapy has not been effective or is unsuitable—eg, in patients with sensitivity against multiple allergens[6]—drug therapy for allergic rhinitis becomes necessary. At present, first-line drug therapy for allergic rhinitis includes topical intranasal steroids and oral antihistamines.[7] However, there is a longstanding discussion on the potential side effects of these medications. Long-term treatment with the available topical steroids might lead to atrophy of the rhinal mucosa and, especially in children, to systemic side effects, and some antihistaminic drugs might cause drowsiness or cardiac side effects, which are reflected by prolongation of the QT-interval. Despite the fact that the new generations of topical steroids and oral antihistamines seem to have considerably more moderate side effect profiles, the need remains for alternative drug therapies that are both safe and efficacious in allergic rhinitis.

One group of potential therapeutic agents are phosphodiesterase 4 (PDE4) inhibitors, which are currently under investigation for use in allergic asthma (for a review, see Giembycz[8]). These drugs exert anti-inflammatory activi-

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Received for publication January 5, 2001; revised July 2, 2001; accepted for publication July 9, 2001.

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0091-6749/2001 $35.00 + 0 1/81/118596
doi:10.1067/mai.2001.118596

ty by blocking the degradation of cyclic adenosine monophosphate in lymphocytes, eosinophils, neutrophils, and monocytes, thus leading to an attenuated release of histamine and leukotrienes as well as of several cytokines, including IL-4, IL-5, IL-10, and GM-CSF.[9-13] These effects have been well established in vitro and in animal models of allergic asthma (for reviews, see Giembycz et al[14] and Torphy[15]).

One such PDE4 inhibitor currently under clinical investigation is roflumilast (3-cyclopropylmethoxy-4-difluoromethoxy-N-[3,5-di-chloropyrid-4-yl]-benzamide; Byk Gulden Pharmaceuticals, Konstanz, Germany).[16,17] Roflumilast exerts selective anti-inflammatory activity in effector cells, such as mast cells, eosinophils, neutrophils, T lymphocytes, and macrophages, as shown in in vitro and in vivo studies. Preclinical animal models have indicated that roflumilast inhibits the inflammatory component of asthma; for example, after oral administration of roflumilast to guinea pigs challenged with ovalbumin, a marked inhibition of the specific allergic reaction could be demonstrated, as assessed by cell and protein influx into bronchoalveolar lavage liquid.[16,17]

In phase I studies, approximately 200 subjects were exposed to oral doses of roflumilast; of these, more than 150 subjects received repeated daily doses of 500 μg/day for durations of up to 28 days. Furthermore, in phase II/III studies, more than 1600 patients with allergic asthma were exposed to roflumilast; of these, approximately 400 patients received 500 μg/day for a total duration of more than 9 months. Approximately 150 of these patients received the same dosage for more than 1 year. With respect to studies of chronic obstructive pulmonary disease, roflumilast has been administered to more than 600 patients; most of them have now been receiving roflumilast for more than 6 months, and approximately 200 of them have been receiving 500 μg/day.

A dose of 500 μg of roflumilast was found to be safe and well tolerated in healthy volunteers included in several phase I studies. In addition, higher doses of up to 5000 μg have been investigated. Given its pharmacokinetic half-life, which is approximately 10 to 15 hours, roflumilast is administered in a once-daily regimen. The fact that a simple treatment schedule improves patient compliance and thus efficacy further favors a once-daily treatment regimen over more complex schedules.

Because PDE4 inhibitors demonstrate anti-allergic and anti-inflammatory properties, they might be of benefit in the management of allergic rhinitis. We therefore conducted a controlled allergen provocation study in patients with histories of seasonal allergic rhinitis, investigating the efficacy of the new PDE4 inhibitor roflumilast.

METHODS

Overall study design

The study was a randomized, placebo-controlled, double-blinded, 2-period crossover trial. This study design allowed for comparison of the 2 treatments with each subject serving as his or her own control. To ensure controlled conditions of antigen exposure, the study was conducted during the allergen-free season, controlled allergen challenge being performed during both treatment periods, as described below. At screening and on each study day, it was confirmed that patients were free of symptoms, including those caused by airborne allergens.

Each patient, randomly assigned to 1 of 2 sequence groups (roflumilast/placebo or placebo/roflumilast), received roflumilast and placebo for 9 days each, according to the treatment crossover design of the study; there was a washout period of at least 14 days between treatments. A washout period of 14 days was chosen to exclude a carryover effect. Because of the half-life of roflumilast (approximately 10 to 15 hours), the minimum washout period required would have been approximately 6 days (10 times the half-life); to be on the safe side, the washout period was prolonged to 14 days.

The maximum concentration of roflumilast (given as the geometric mean with 68% range) is 3.845 (3.013 to 4.907) μg/L when the subject has eaten (breakfast) and 6.517 (4.979 to 8.531) μg/L when the subject has fasted. Because the time of maximum concentration of roflumilast is approximately 2 hours, controlled intranasal allergen provocation with commercially available pollen extracts was performed on treatment days 3 through 9, each time approximately 2 hours after study drug administration. Five and 30 minutes after allergen challenge, rhinal airflow was measured by means of rhinomanometry and the subjective symptoms obstruction, itching, and rhinorrhea were assessed by means of a standardized visual analog scale, as described below. The 2 different assessment time points were chosen because of the known variability in the time course of a patient's response to allergen challenge.

Subjects

Twenty-five subjects (16 male and 9 female), each with a history of seasonal allergic rhinitis but asymptomatic at screening, were enrolled and included in the safety analysis. A total of 24 subjects (12 of each sequence) completed the study (median age, body weight, and height were 27 years, 76 kg, and 181 cm, respectively) and were available for efficacy analysis. One subject, having received 4 doses of roflumilast, was prematurely withdrawn by his own decision on day 4 of the first treatment period.

Inclusion criteria included the following: a history of seasonal allergic rhinitis (as stated by the patient); positive skin prick test results (see below); a reduction of at least 33% in mean rhinal airflow after allergen provocation, as measured by rhinomanometry; age between 18 and 45 years. Exclusion criteria included the following: any active disease or relevant abnormalities, as assessed in a prestudy examination; abnormal ear, nose, and throat status (eg, relevant septum deviation); symptoms of allergic rhinitis at screening; history of asthma attacks or severe anaphylactic reactions; any history of drug allergy; use of any medication during the 2 weeks preceding the study; topical or systemic anti-allergic medication use, including steroids or decongestive nose drops, during the 4 weeks preceding the study. Subjects were not allowed to smoke more than 10 cigarettes per day or to drink alcohol or coffee to excess (>20 g of alcohol or >5 cups of coffee per day). In addition, the following exclusion criteria were taken into account for women: no reliable contraception in the cycle before and after the study as well as during the study period (only intrauterine devices or registered hormonal contraceptives were allowed); pregnancy; lactation period.

Screening procedure and allergen provocation

Each of the subjects had a comprehensive medical examination performed within the 2 weeks before study inclusion. This screening examination consisted of the following: the taking of a medical history; physical examination, including nose and throat; 12-lead electrocardiography; body temperature recording; clinical laboratory

parameter determination; and a skin prick test through use of a standard battery of 20 common aeroallergens extracts*. In case of a positive skin prick test result (defined in comparison with the histamine and a negative control as a wheal diameter 3 mm larger than that of the negative control) to the pollen preparation, active anterior rhinomanometry was carried out to obtain a baseline rhinal flow value; this was followed by a controlled antigen exposure with commercially available pollen extracts (Allergopharma Joachim Ganzer KG).

Allergen challenge was done by spraying 2 puffs of the pollen suspension in each nostril. For antigen delivery, metered-dose sprays were used to provide 0.05 mL atomized solution per puff. The allergen concentration was 5000 standardized biological units/mL. The pollen extracts were selected individually for each subject by choosing the 1 or 2 allergens that had evoked a major reaction in the skin prick test during the screening examination. For particular subjects, the same allergens were used during the entire study. These were grass in 8, rye in 7, birch in 5, and hazel in 2 patients. Two patients received hazel and grass. Nasal congestion was objectively assessed by standardized rhinomanometry through use of commercially available equipment (Allergopharma Joachim Ganzer KG).

Rhinal airflow was determined as the sum of the values of both nostrils obtained at a pressure difference of 150 Pa. The rhinal flow values at 5 and 30 minutes after allergen provocation were averaged before further analysis. The percentage change from the predose value served as inclusion criterion. A subject was included in the study only if the rhinal flow decreased by at least 33% after allergen provocation at screening.

Study medication

Roflumilast at a dose of 500 μg once daily (2 tablets of 250 μg each) and placebo were administered in tablets of identical appearance to ensure that proper blinding was maintained.

Course of the study

In each treatment period, the study medication was administered on each of 9 consecutive days (study days 1 through 9) at approximately 8:00 AM under the supervision of the investigator. Two tablets, each containing 250 μg of roflumilast or placebo, were administered with 200 mL tap water after the subject ate a continental breakfast. Controlled antigen challenge was performed at 10:00 AM for 7 consecutive days (study days 3 through 9) in each study period by spraying 2 puffs of the pollen suspension into each nostril, as described for the screening visit. Five and 30 minutes after each allergen provocation, subjective nasal symptoms—obstruction, itching, and rhinorrhea—were evaluated and rhinomanometric measurements were performed.

The 3 subjective symptoms were assessed by means of a standardized visual analog scale (10 cm), the readings ranging from "Not in existence" to "Very strong." Considering the severity of the symptom, the subject indicated the corresponding position on the scale, and the distance between "Not in existence" and the position indicated (in centimeters) represented the symptom score assigned (a value between 0 and 10). At each time point, the assessment of the subjective rating (both nostrils included) was done before the rhinomanometric measurements, because nasal symptoms can be temporarily influenced by the local manipulation imposed during measurement.

The safety of roflumilast was assessed by continuous monitoring of adverse events and by additional safety measurements made at a final visit. The poststudy examination, including an ear, nose, and throat examination, was performed within 2 weeks of completion of the study.

Biostatistical methods

The primary variable for confirmative biostatistical analysis was rhinal airflow, as determined by rhinomanometry. The comparison of the rhinal airflow values was done by means of ANOVA for the 2-treatment, 2-period crossover design after logarithmic transformation. A separate ANOVA was performed for each of days 3 through 9. Treatment differences for these 7 days were tested by means of a stepwise closed testing procedure without need to adjust the α-level[18]; consecutive comparisons were performed beginning with day 9. If the difference was found to be significant, testing was done for day 8. This procedure was followed in reverse order until day 3. In case of a nonsignificant result, subsequent comparisons were not performed. For the sake of consistency with the multiplicative model, geometric means were calculated for rhinal airflow.

Secondary variables included the subjective nasal findings (obstruction, itching, and rhinorrhea). These variables were analyzed as described above for the rhinal flow, except that an additive model was used.[19] For itching and rhinorrhea scores, the evaluation was performed separately for the time points 5 minutes and 30 minutes after allergen provocation, because itching and rhinorrhea scores were generally lower at 30 minutes than at 5 minutes (in contrast to rhinal airflow and obstruction score, for which similar values were obtained at the 2 time points). Because of their exploratory nature, no adjustment of the α-level was made for the testing of these multiple secondary variables.

The sample size of 24 subjects was chosen to provide 80% power in detecting a 20% difference in rhinal flow on roflumilast versus placebo at a significance level of 5% (2-sided), assuming an intrasubject coefficient of variation of approximately 20%, as indicated in a previous study.[20]

Organization of the study

The study was conducted in accord with the revised Declaration of Helsinki, the German Drug Law, and the requirements of Good Clinical Practice.[21] Ethical approval was obtained from the independent Ethics Committee of the Faculty of Clinical Medicine Mannheim of the University of Heidelberg. The clinical portion of the study was conducted at the Mannheim University Hospital, Germany, and sponsored by Byk Gulden Pharmaceuticals.

Results

Efficacy analysis

Roflumilast consistently provided greater improvement than placebo in geometric mean rhinal flow on all allergen provocation days (Fig 1). In comparison with placebo, roflumilast exhibited a statistically significant ($P = .007$) greater improvement (15%) on day 9. Because the difference on day 8 did not reach statistical significance, data from the earlier days (3-7) were not subjected to further hypothesis testing.

This finding was confirmed by the results of the subjective obstruction score (Fig 2). Roflumilast demonstrated a significant difference in comparison with placebo ($P < .05$; 2-sided) from study day 4 to study day 9. The subjective impression of symptom alleviation in comparison with placebo was highest on day 5 (with a reduction of 42% in the airway obstruction symptom score). Inasmuch as the subjective feeling of obstruction is caused by multiple factors in addition to the measured nasal flow, this

*The following allergens were used: maple, birch, early flowers, grain, grass (2 different preparations), rye, herbs, bushes, nettle, hazel, *Aspergillus fumigatus*, *Candida albicans*, *Cladosporidium*, *Penicillium notatum*, *Trichosporum*, mite (2 preparations), and animal epithelia (2 preparations; Allergopharma Joachim Ganzer KG, Reinbek, Germany).

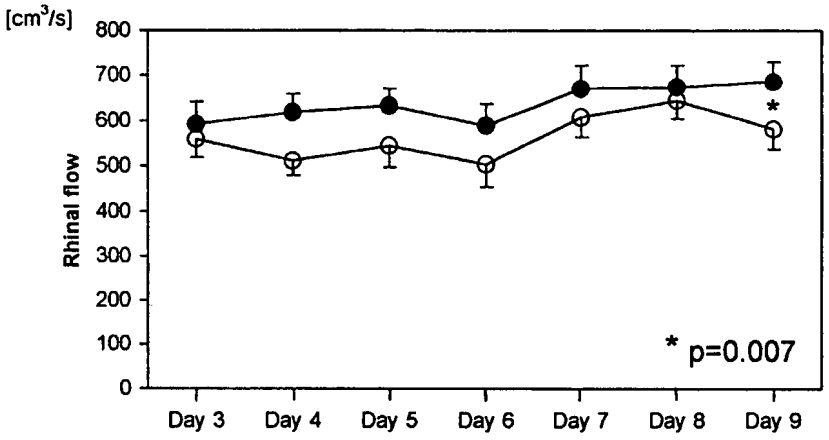

FIG 1. Time course of airway obstruction, determined objectively by rhinomanometry (geometric mean and SEM; n = 24) after controlled allergen challenge (5 and 30 minutes averaged) during treatment (days 3 to 9) with 500 μg of roflumilast (●) and placebo (○).

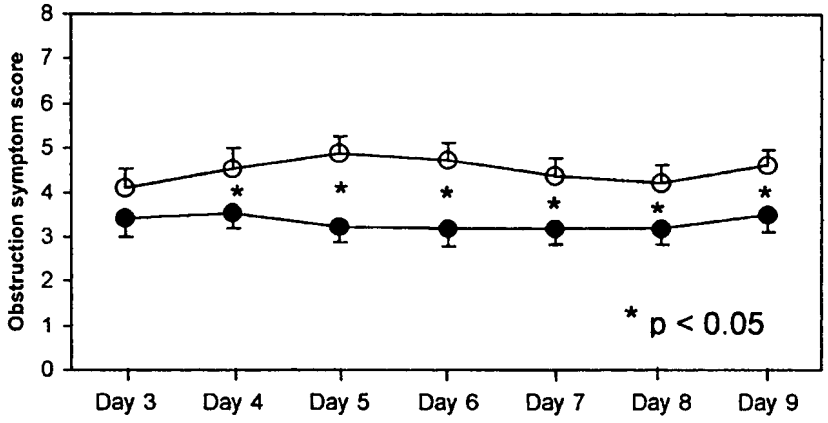

FIG 2. Time course of airway obstruction symptom score (mean and SEM; n = 24) after controlled allergen challenge (5 and 30 minutes averaged) during treatment (days 3 to 9) with 500 μg of roflumilast (●) and placebo (○).

not only confirms but expands the relevance of the results of the objectively determined rhinal flow.

On all study days, the mean itching and rhinorrhea scores during roflumilast treatment were below those obtained during placebo treatment at either measuring time point. Mean itching and rhinorrhea scores observed at 5 minutes were higher than those observed at 30 minutes after allergen challenge, independently of whether the subjects received placebo or the active compound. With respect to itching, roflumilast treatment demonstrated a statistically significant difference in comparison with placebo at both time points on day 9 and at 5 minutes on all study days (Fig 3). Furthermore, with respect to rhinorrhea, roflumilast treatment demonstrated a statistically significant difference in comparison with placebo at 5 minutes on days 8 and 9 (Fig 4).

Safety

In general, treatment with roflumilast was found to be safe and well tolerated. No clinically relevant abnormalities in physical examination or clinical laboratory findings were noted. During treatment with roflumilast, headache was reported most frequently (7 of 25 subjects), followed by nausea (5 of 25) and dizziness (4 of 25). Most of the adverse events were mild; only 2 cases of headache were classified as moderate by the investigator. All of these events subsided spontaneously during continuous treatment. No serious adverse event occurred. The duration of the adverse events varied between 1 hour and 10 days. In the placebo group, only 2 adverse events were reported (dizziness and hiccups).

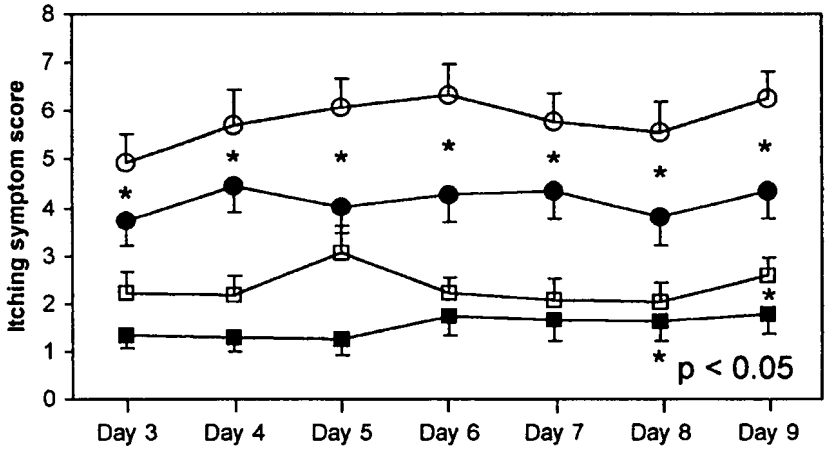

FIG 3. Time course of itching score (mean and SEM; n = 24) after controlled allergen challenge (separately for 5 and 30 minutes) during treatment (days 3 to 9) with 500 μg of roflumilast (5 minutes, ●; 30 minutes, ■) and placebo (5 minutes, O; 30 minutes, □).

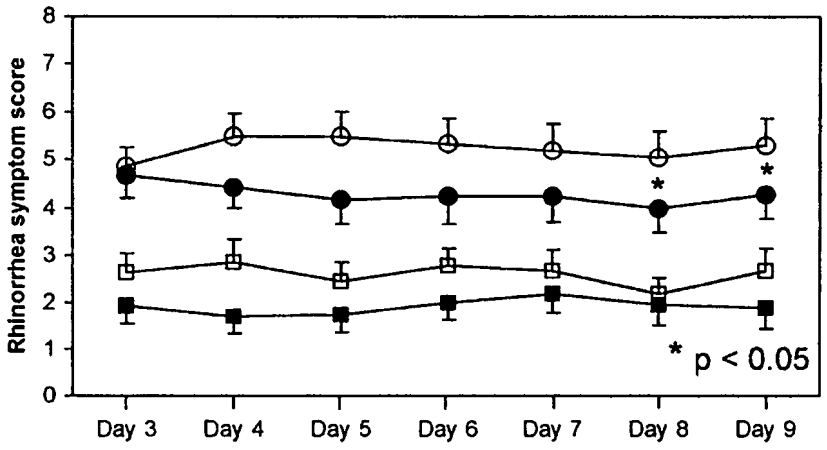

FIG 4. Time course of rhinorrhea score (mean and SEM; n = 24) after controlled allergen challenge (separately for 5 and 30 minutes) during treatment (days 3 to 9) with 500 μg of roflumilast (5 minutes, ●; 30 minutes, ■) and placebo (5 minutes, O; 30 minutes, □).

DISCUSSION

This is the first study demonstrating the efficacy of a PDE4 inhibitor in allergic rhinitis. The results clearly show that the PDE4 inhibitor roflumilast is effective in relieving the symptoms of allergic rhinitis.

This effect might be mediated by the immunomodulatory and anti-inflammatory action of specific inhibitors of the PDE4 isoenzyme, which is prevalent in white blood cells.[22] Specific inhibitors of PDE4 are currently under investigation, mainly for use in chronic obstructive pulmonary disease and asthma (for review, see Giembycz[8] and Schudt et al[23]). Inhibition of PDE leads to increased intracellular cyclic adenosine monophosphate levels, which in turn suppress histamine release from mast cells and the release of several cytokines and chemokines that are involved in allergic inflammation, such as IL-1β,[9] IL-4,[10] IL-5, IL-10, IL-8,[11] TNFα[9] and leukotrienes.[12,13] In particular, the recruitment of eosinophils is decreased because of the reduced release of cytokines, especially IL-5, and probably also because of the lower expression of adhesion molecules on endothelial cells.[24] PDE4 inhibitors might also play a role in T_H1-mediated autoimmune diseases because of their inhibition of T-cell proliferation and a shift of the cytokine profile toward a T_H2-phenotype,[25] which is accompanied by the suppression of MHC-II activated immunity.[26] In contrast to that of the glucocorticoids, the activity of PDE4 inhibitors appears to be even more pronounced in patients with a predisposition to atopic reactions.[27]

It was postulated that PDE4 inhibitors might be effective in the treatment of allergic rhinitis, inasmuch as the reaction causing allergic rhinitis uses, at least in part, the

immunologic pathways that are used by allergic asthma. The results of the current study support this hypothesis. An increase of the rhinomanometrically measured airflow was found as early as 3 days after treatment start, reaching statistical significance in comparison with placebo after 9 days. The difference in subjective impression of obstruction alleviation became significant on the fourth treatment day. Furthermore, statistically significant relief in comparison with placebo regarding the allergic symptoms itching and rhinorrhea was demonstrated.

Our study design might be seen as artificial. A more "natural" approach in pharmacodynamic studies is evaluation of the efficacy of a newly developed antiallergic drug in patients with allergic rhinitis during the pollen season, using natural allergen exposure.[28] Use of this method would have provided the advantage of evaluating nasal symptoms under the patients' usual living conditions. However, the concentration of airborne pollen grains might then have varied during the trial period, and this in turn would have influenced the study results. With controlled antigen challenge, an improved standardization of the study conditions could be achieved. To avoid additional exposure to airborne allergens, any such trial should be performed during the allergen-free season.[29]

During the clinical development of a new antiallergic drug such as roflumilast, the first exploratory study might be conducted during the allergen-free season through use of controlled antigen delivery, inasmuch as the highly standardized conditions allow a relatively small sample size for such a "proof-of-concept" trial. In later phases, when the efficacy is to be confirmed in a larger number of patients in phase III trials, the studies should be conducted during the pollen season under the patients' usual living conditions. This is also required by agencies considering registering of the drug.

In the present study, a spray was used to deliver a defined amount of pollen extract into the nostrils. Although favorable experiences with environmental exposure units have been reported,[30,31] the method is expensive and requires special facilities. In contrast to the environmental exposure unit, in which all subjects are exposed to the same aeroallergens, the method of intranasal allergen provocation allows the individual selection of a particular allergen to be used for provocation, an approach that we considered at least equally valid for the type of study conducted.

The rationale for performing multiple challenges in this study was to simulate the conditions of natural allergen exposure over prolonged periods; furthermore, we were thereby able to determine the onset of action of roflumilast treatment. This approach had proved to be useful in a previous trial reported by us, in which the onset of efficacy of an intranasally given steroid was determined.[20] In contrast to the previous investigation, a pretreatment with the trial medication was performed on the 2 days before the start of the allergen challenge, because it was hypothesized that roflumilast would already show activity on the third treatment day (preventive model); this, however, was not the case.

Because of the repeated activation, a priming effect could have occurred during the study periods. However, as seen in Fig 1, rhinal airflow rose in the placebo period on days 7 through 9 in comparison with days 3 through 6, suggesting that no priming effect occurred. It is probable that once-daily exposure to the allergen is not sufficient to induce a priming effect.

Determination of nasal air flow as a sum after allergen release to both nostrils is not a standard measure in the clinical setting. It was chosen in this study to reduce intraindividual variability, inasmuch as the effects of the physiological cyclic swelling of the nasal mucosa would be expected to average out. Moreover, the method of controlled intranasal allergen challenge and the subsequent evaluation of drug efficacy by concomitant use of anterior rhinomanometry and a symptom score had been shown to be reliable and valid in a previous trial.[20] It was found that the clinical efficacy of topical steroids administered as nasal sprays can be quantitatively assessed by rhinomanometry.[32] The use of symptom scores is likewise a commonly accepted method.[33] Although the results of subjective and objective assessment of nasal obstruction have been shown to be significantly correlated,[34] there have also been reports that the rhinomanometric measurements of nasal airflow do not always reflect the patients' sensation of nasal obstruction.[35] Therefore, the present study evaluated the intensity of subjective nasal symptoms in parallel with rhinomanometric airflow measurements, because this complementary information might provide a more reliable assessment of nasal symptoms than the objective measurements alone. This study design was considered particularly useful for a first efficacy study on a new antiallergic compound and was thus chosen for the current trial with roflumilast.

In conclusion, this study provides evidence that oral treatment with roflumilast is an effective antiallergic therapy in allergic rhinitis. The improvement in airway obstruction seen after oral administration of the PDE4 inhibitor roflumilast was slightly inferior to the treatment effect seen in our previous study of comparable design, which evaluated the intranasal administration of the new topical steroid ciclesonide.[20] Direct comparison of the results of the present study with literature data obtained with other classes of drugs is hampered by substantial differences in the study designs. Several points have to be taken into consideration—eg, primary variables (objective/subjective assessment of symptoms), sample size, allergen exposure (natural/controlled), and overall statistical plan (parallel-group design/crossover design). The efficacy results of the present study are noteworthy, but given that our findings were obtained in a relatively small group of patients (N = 24), further studies will be required to evaluate the efficacy of roflumilast in patients with allergic rhinitis under more natural environmental and common clinical practice conditions. Moreover, comparative studies against established treatment regimens will be necessary to further specify the place that PDE4 inhibitors might take in this indication field.

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Suppression of Acute Lung Injury in Mice by an Inhibitor of Phosphodiesterase Type 4

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The present study has investigated the therapeutic potential of a type 4 phosphodiesterase (PDE) inhibitor, rolipram, in experimental lung injury. Acute lung injury was induced in the mouse by combined treatment with lipopolysaccharide (LPS; 10 mg/kg, i.v.) and zymosan (3 mg/kg, i.v.), and assessed using extravascular albumin accumulation; neutrophil sequestration in pulmonary capillaries was also measured. The results show that pretreatment with rolipram (5 mg/kg, i.p.) was protective against the induction of lung injury by combined LPS and zymosan; extravascular albumin accumulation was reduced by 89% and neutrophil sequestration in lung tissue, as assessed by lung myeloperoxidase (MPO) activity was reduced by 75%. Pretreatment with rolipram also attenuated increases in serum tumor necrosis factor alpha (TNFα) levels induced by LPS and zymosan treatment, measured after 2.5 h. The role of endogenous TNFα in the induction of lung injury was therefore assessed. Blockade of endogenous TNFα by treatment with the soluble receptor p55-IgG fusion protein or an anti-murine TNFα monoclonal antibody, TN3.19.12, had no protective effect against LPS and zymosan-induced lung injury. This suggests that there is a disassociation between TNFα production and the induction of injury in this model. Administration of rolipram after LPS and before zymosan treatment obliterated the increase in pulmonary vascular permeability, but its effect on sequestration of neutrophils in pulmonary microvessels, as measured by MPO, was less marked. The results of the present study suggest that use of agents such as rolipram that inhibit PDE4 may have a therapeutic role in treatment of acute lung injury, since we have shown that it is effective in attenuation of neutrophil activation even after sequestration. However, its effect appears to be independent of TNFα inhibition.
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Acute lung injury or acute respiratory distress syndrome (ARDS) is associated with a spectrum of medical conditions and is a manifestation of acute vascular disruption. The sequestration of neutrophils in the pulmonary microcirculation and their activation appears to be a key event in the development of the lung injury. The sequestered neutrophils, when activated, are a source of proteases, reactive oxygen species and inflammatory mediators (1). These products can contribute to pulmonary vascular endothelial cell and alveolar epithelial cell damage and promote increased pulmonary vascular permeability and edema formation, features of pulmonary dysfunction (1). The ensuing impaired gaseous exchange can be a direct cause of mortality. The onset of ARDS is often an early symptom of multiple organ failure associated with sepsis, and this is associated with elevated blood levels of endotoxin or lipopolysaccharide (LPS). LPS has therefore been implicated as an important inducer of lung injury (2) and experimentally, endotoxin or LPS has been used to induce acute lung injury in animals (3–7). LPS has many proinflammatory actions in the lung, including the induction of neutrophil sequestration in pulmonary capillaries, upregulation of cell adhesion molecules on endothelial cells (8–10), and the promotion of cytokine synthesis and release from alveolar macrophages and endothelial cells (11, 12). One of the principal cytokines induced by LPS is tumor necrosis factor alpha (TNFα). This cytokine has been implicated as

(Received in original form February 3, 1997 and in revised form June 30, 1997)

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Abbreviations: acute respiratory distress syndrome, ARDS; cyclic adenosine 3′5′-monophosphate, cAMP; human serum albumin, HSA; lipopolysaccharide, LPS; monoclonal antibodies, mAb; myeloperoxidase, MPO; phosphodiesterase, PDE; tumor necrosis factor alpha, TNFα; tumor necrosis factor receptor, TNFR.

a mediator of the pathologic changes encountered in septic shock, because TNFα levels are elevated in the plasma, bronchoalveolar lavage (BAL) fluid, and lung tissue of septic patients with ARDS (13–15). Administration of TNFα itself to animals can induce neutrophil sequestration in pulmonary capillaries as well as their activation (16–18) and can cause pulmonary damage *in vivo* (19).

It is conceivable, therefore, that attenuation of LPS-induced lung injury may be achieved by the inhibition of TNFα production (or action) *in vivo*. Agents which increase intracellular cyclic adenosine 3′5′-monophosphate (cAMP), such as prostaglandin E_1 and phosphodiesterase (PDE) inhibitors or cAMP analogues, all inhibit TNFα production both *in vitro* (20–22) and *in vivo* (23). The intracellular levels of cyclic nucleotides, including cAMP, are regulated by a family of PDE enzymes which degrade them and render them biologically inactive (24). Inhibition of the PDE enzymes results in an accumulation of intracellular cAMP, which leads to a suppression of neutrophil activity, including chemotaxis, degranulation, and the respiratory burst (25–27). The nonspecific PDE inhibitor pentoxifylline has been shown to be protective in lung injury induced by endotoxin and by TNFα in dogs and guinea pigs (28, 29). Since the main PDE isoenzyme in cells involved in the inflammatory process is type 4, studies have investigated the effects of specific inhibitors of PDE4 in LPS-induced organ injury. In one study, the specific PDE4 inhibitor rolipram was reported to attenuate LPS-induced mortality and gross pulmonary injury in rats, and this was attributed to suppression of the increases in serum TNF levels (30). However, the role of endogenous TNFα in mediating the induction of lung injury remains unclear and there seems to be contention as to whether TNFα plays a role in LPS-lung injury.

The aim of the present study, therefore, was to ascertain the effect of PDE4 inhibition on induction of lung injury and to determine possible modes of action. We have previously described a murine model of acute lung injury, induced by combined treatment with LPS and zymosan (31), where we have observed that the induction of injury is dependent on the activation of sequestered neutrophils. We have therefore investigated the effect of the specific PDE type 4 inhibitor rolipram on lung injury, as assessed by increases in pulmonary vascular permeability and neutrophil sequestration, as well as measuring levels of TNFα in serum. In addition, we have assessed the effect of neutralization of endogenous TNFα on lung injury by use of a soluble TNFα receptor protein and a specific chimeric antibody against murine TNFα.

Materials and Methods

Induction of Experimental Acute Lung Injury

Lung injury was induced in BALB/c female mice (18–20 g, Harlan Olac, Bicester, UK) by injection of LPS from *Escherichia coli* 0111:B4 (Sigma, Poole, UK) at a dose of 3 mg/kg (i.v.). This dose of LPS has been shown to induce neutrophil sequestration in the lung at 2 h, but does not result in detectable lung injury (31). Control mice received an i.v. injection of saline (7 ml/kg). After 2 h, zymosan A (10 mg/kg) from *Saccharomyces cerevisiae* (Sigma, UK), or saline in control animals, was then injected i.v. simultaneously with ^{125}I-human serum albumin (HSA) (approximately 250 nCi/animal; Amersham International, Little Chalfont, UK). Extravascular ^{125}I-HSA was used as a measure of increased microvascular permeability in lung tissue and its accumulation was measured after 30 min. At this time point, ^{131}I-HSA (approximately 500 nCi/animal), prepared according to the chloramine T method (32), was injected i.v. and allowed to circulate for 5 min. ^{131}I-HSA was used to quantify the intravascular volume of the lung. The mice were then given sodium pentobarbitone to induce deep anesthesia and were killed by exsanguination. A blood sample was collected into heparin and the plasma fraction was prepared. The lungs were exposed, removed *en bloc* and the activities of ^{125}I-HSA and ^{131}I-HSA in whole lungs were counted in a gamma counter and compared with those in the plasma. The volume of extravascular albumin accumulated in lung tissue was then calculated by subtracting the tissue ^{131}I-HSA plasma volume from the ^{125}I-HSA plasma volume and was expressed as microliters of plasma equivalents retained in whole lung tissue.

In separate groups of animals, zymosan alone (10 mg/kg) was injected i.v. together with ^{125}I-HSA and extravascular albumin accumulation was assessed after 30 min in the same manner described above.

Treatment with Rolipram

Rolipram was a gift from Dr. J. Fozard, Sandoz, Basel, Switzerland. It was dissolved in ethanol and further diluted in saline to a final concentration of 0.5 mg/ml in no more than 2.5% ethanol. Rolipram was injected i.p., at doses of 1 mg/kg and 5 mg/kg, 30 min prior to further i.v. treatment with combined sequential LPS and zymosan administration. These doses were chosen since they have been shown to be effective in LPS-induced mortality in rats (29) and to reduce neutrophil recruitment into the peritoneal cavity of mice (33). In a separate group of animals, rolipram (5 mg/kg) was administered after LPS treatment and 30 min prior to subsequent zymosan.

Treatment with TNFR-IgG Fusion Protein and mAb TN3.19.12

The soluble human TNFα receptor (p55)-IgG fusion protein (TNFR-IgG) was a gift from Drs. Scallon and Ghrayeb, Centocor Inc. (Malvern, PA). Neutralization of endogenous TNFα was achieved by i.p. administration of TNFR-IgG at a dose of 5 mg/kg 6 h before subsequent LPS and zymosan treatment. Lung injury was measured as previously described. The dosing regimen has been shown to be protective against LPS-induced mortality and attains complete neutralization of circulating TNFα in mice (34).

In addition, the anti-TNFα monoclonal antibody (mAb) TN3.-19.12, a gift from Dr. R. Foulkes, Celltech, Slough, UK, was tested on LPS plus zymosan-induced lung injury. TN3.19.12 is a murine/hamster chimeric antibody directed against murine TNFα with hamster variable regions and murine heavy and light chain constant regions. Mice were injected i.v. with TN3.19.12 (30 mg/kg) 1 h before treatment

with LPS and zymosan, and lung injury was measured as previously described. This dose of mAb has been previously shown to inhibit LPS-induced mortality in mice by 90% (35).

Histology

In appropriate experiments, after exsanguination, the lungs were exposed and a catheter was secured into the trachea in order to inflate the lungs *in situ* with 10% neutral buffered formalin (pH 7.0), until the pleural margins were sharp. The lungs were then removed *en bloc* and further fixed by immersion in formalin until processing to paraffin wax. Sections (5–6 μm) were cut and stained with hematoxylin and eosin for assessment of leukocyte sequestration.

Tissue Extraction and Measurement of Myeloperoxidase Activity

The extent of neutrophil sequestration in whole lung tissue was measured by assaying myeloperoxidase (MPO) activity (36). The lungs of animals that had received LPS plus zymosan with or without rolipram treatment, zymosan alone with or without rolipram treatment, or saline were removed and frozen in liquid nitrogen. Upon thawing, the tissue was homogenized in 0.2% NaCl buffer (pH 4.7) and centrifuged at 260 × *g* for 10 min. The supernatant was isolated and ultracentrifuged at 100,000 × *g* for 60 min, whereupon the pellet was resuspended in hexadecyltrimethyl-ammonium bromide. MPO activity in the resuspended pellet was assayed by measuring the change in optical density (O.D.) at 690 nm using tetramethylbenzidine (1.6 mM) and H_2O_2 (0.3 mM). Results were expressed as change in absorbance (O.D.) per gram of lung tissue.

MPO activity was also assayed in whole lung tissue taken from animals pretreated with either anti-TNF treatment, i.e., TNFR-IgG and TN3.19.12.

Measurement of Serum TNFα Levels

Serum samples were prepared from blood taken from control saline-treated mice, LPS and zymosan-treated mice, and mice pretreated with rolipram prior to combined LPS and zymosan administration. TNFα levels were measured using a sandwich ELISA kit purchased from Endogen (Bradsure Biologicals, Loughborough, UK). This kit is reported to detect mouse TNF levels at a concentration of > 10 pg/ml.

Biological activity of TNFα in the serum of mice treated with the anti-TNF treatments was measured by assessing cytotoxicity of the serum on WEHI 164 cells (37). The WEHI assay was kindly performed by Dr. D. Butler at the Kennedy Institute of Rheumatology (London, UK) as previously described (38).

Statistics

All data are expressed as mean ± SEM. One-way analysis of variance (ANOVA) was used for analysis of the data groups. The Student-Newman-Keuls correction factor for multiple comparisons was used as a post test. Differences were considered significant when probability values were 0.05 or less.

Results

Effect of Rolipram Pretreatment on Lung Injury Induced by Combined LPS and Zymosan

Treatment with LPS followed by an i.v. injection of zymosan 2 h later resulted in a significant ($P < 0.01$) increase in extravascular albumin accumulation in lung tissue when compared with control saline-treated animals (Figure 1a).

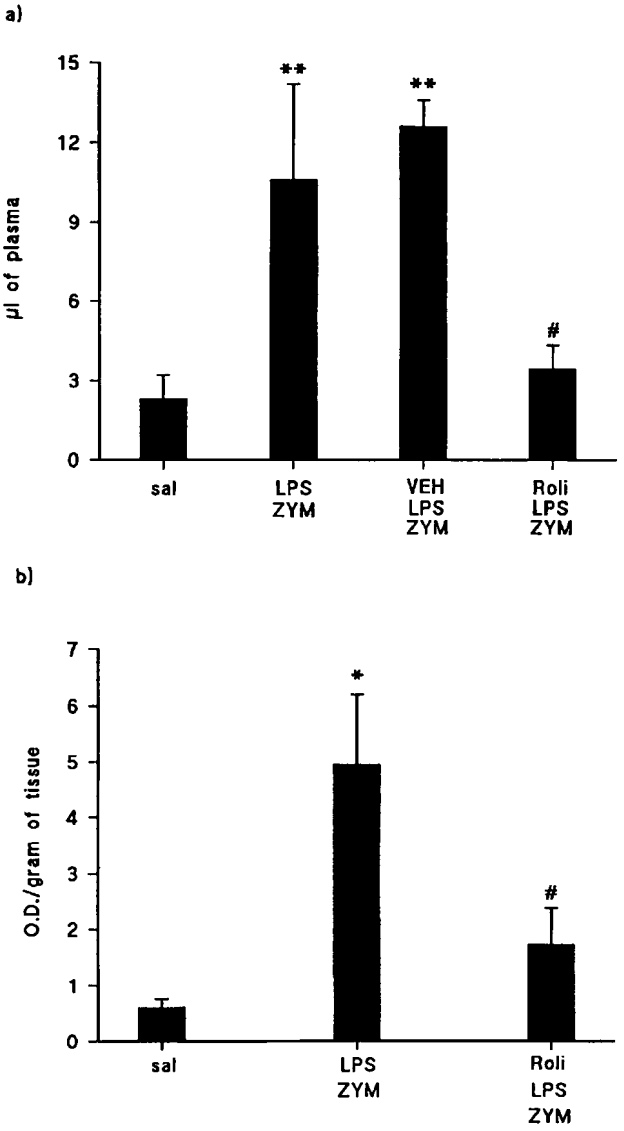
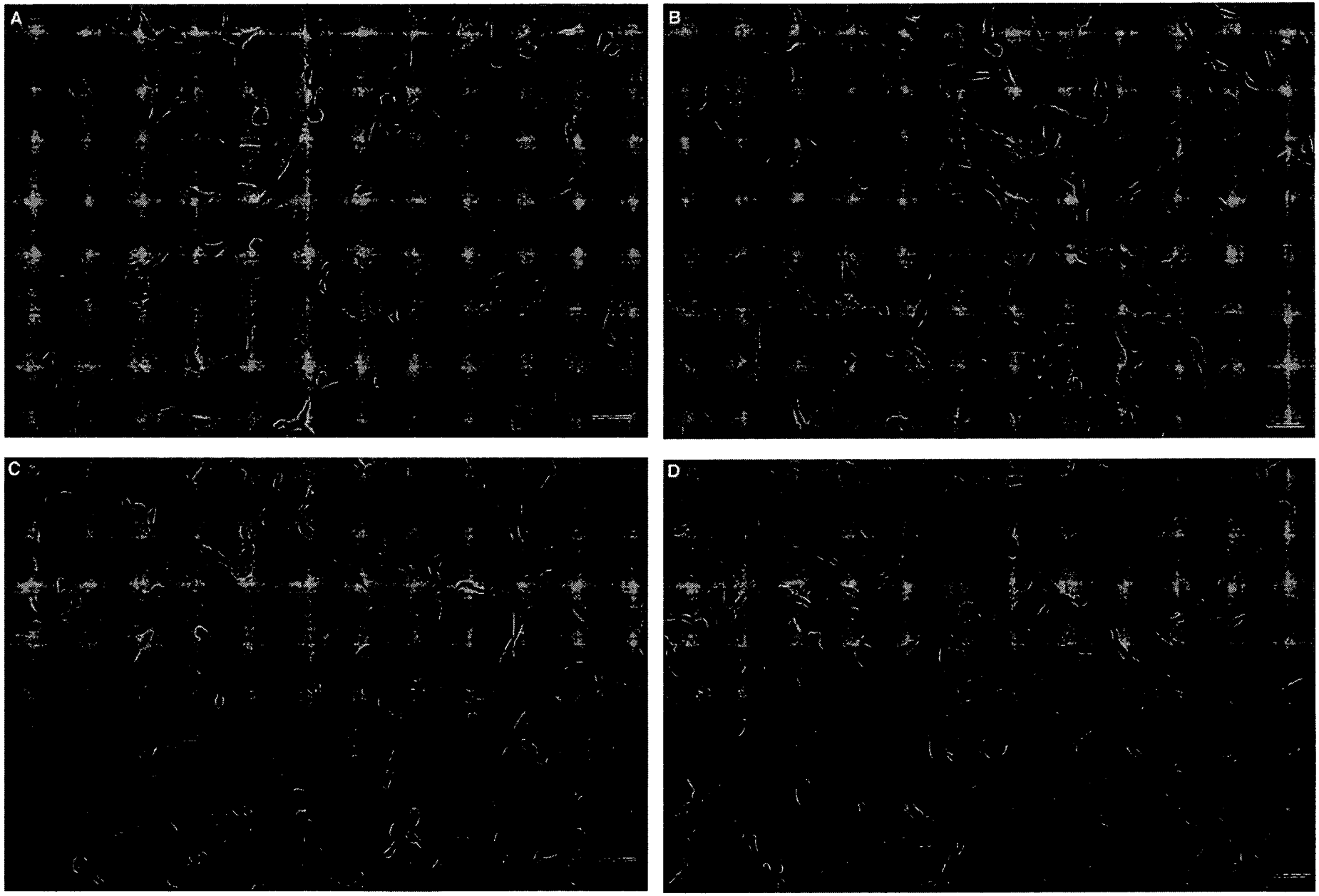


Figure 1. Effect of rolipram on acute lung injury induced by combined LPS and zymosan treatment. (*a*) Pretreatment with rolipram (5 mg/kg, i.p.) prior to administration of LPS and zymosan led to a decrease in the amount of extravascular ^{125}I-HSA measured in mouse lung tissue. Results are expressed as mean ± SEM of 3–6 animals, where $^{**}P < 0.01$ compared with saline and $^{\#}P < 0.01$ compared with LPS and zymosan. (*b*) Pretreatment with rolipram prior to LPS and zymosan significantly reduced MPO levels, when compared with animals treated with LPS and zymosan alone. Results are expressed as mean ± SEM of 4 animals where $^{*}P < 0.05$ compared with saline and $^{\#}P < 0.05$ compared with LPS and zymosan.


A
B
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D

These data are consistent with our earlier findings (31). The response to LPS and zymosan was not significantly modified by pretreatment with the rolipram vehicle (2.5% ethanol, 10 ml/kg; Figure 1a). However, 30 min pretreatment with rolipram (5 mg/kg) before LPS and zymosan resulted in an 89% inhibition of extravascular albumin accumulation ($P < 0.05$; Figure 1a). A lower dose of rolipram (1 mg/kg) had a partial, but nonsignificant, effect on LPS and zymosan-induced injury (7.3 ± 2.8 μl, $n = 5$) and all further studies were therefore conducted using 5 mg/kg.

The magnitude of neutrophil sequestration was quantified by assaying MPO activity in lung tissue. Combined LPS and zymosan treatment resulted in MPO activity increasing approximately 8-fold when compared with that in lung tissue taken from saline-treated controls (Figure 1b). Pretreatment with rolipram significantly inhibited this increase by approximately 75% ($P < 0.05$).

In addition, lung tissue was examined by light microscopy. Treatment with combined LPS and zymosan led to a marked and diffuse sequestration of neutrophils within pulmonary capillaries when compared with saline-treated controls (Figures 2A and 2B). The neutrophils appeared to be intravascular. There was no evidence of intra-alveolar edema formation, which is consistent with our previous finding that the extravascular lung water measured in control saline-treated and LPS and zymosan-treated mice is not significantly different (39). In contrast, there was an apparent decrease in neutrophil recruitment in the lung sections taken from animals treated with rolipram prior to receiving LPS and zymosan (Figure 2C), thus confirming the results obtained for MPO assay.

Effect of Rolipram Pretreatment on Serum TNFα Levels

Levels of TNFα were measured in the serum obtained from saline and LPS and zymosan-treated mice. TNFα levels in saline-treated animals were below the detection limits of the kit (i.e., < 10 pg/ml). LPS and zymosan treatment resulted in a substantial increase in TNFα serum levels to 112 ± 41 pg/ml ($n = 3$) at 2.5 h. In animals pretreated with rolipram, the increases in serum TNFα induced by subsequent LPS and zymosan were diminished and the levels were around 25% of those detected in the mice not receiving rolipram (28 ± 8 pg/ml; $P = 0.051$).

Effect of Blockade of TNFα on LPS and Zymosan-induced Lung Injury

Since lung injury was attenuated by PDE4 inhibition and this was associated with a reduction in serum TNFα levels, it was hypothesized that neutralization of endogenously liberated TNFα would lead to an attenuation of the induction of injury. We therefore assessed the effect of TNFα neutralization by pretreatment with TNFR-IgG. However, as can be seen in Figure 3a, 6 h pretreatment with TNFR-IgG (5 mg/kg, i.p.) had no significant effect on LPS and zymosan-induced lung injury, which remained significantly ($P < 0.01$) elevated above saline-treated controls.

In addition, the specific anti-murine TNFα mAb TN3.-19.12 was used at a dose (30 mg/kg, i.v.) which has been shown to be efficacious in reducing LPS-induced mortality in mice. Similar to the findings with TNFR-IgG, pretreatment with TN3.19.12 did not alter extravascular albumin accumulation in response to LPS and zymosan treatment, which remained significantly ($P < 0.01$) elevated above saline-treated controls.

Neutrophil sequestration in lung tissue was quantified in animals pretreated with both the mAb TN3.19.12 and the fusion protein TNFR-IgG. MPO activity was increased by approximately 4-fold as a result of LPS and zymosan treatment and was not significantly altered by pretreatment with either TNF blocking treatment (Figure 3b).

The efficiency of anti-TNFα treatments in neutralizing serum TNFα activity was assessed in the WEHI assay. Compared with LPS plus zymosan-treated animals with serum TNFα activity of 196 U/ml, TNFα activity in the serum of animals pretreated with either the fusion protein TNFR-IgG or anti-TNFα mAb was reduced by approximately 99% and was not significantly different from the levels measured in saline-treated controls (0.6 U/ml).

Effect of Delayed Treatment with Rolipram on Lung Injury Induced by Combined LPS and Zymosan

Since the anti-inflammatory effects of rolipram appeared to be independent of endogenous TNFα, we assessed the effect of PDE4 inhibition on the capacity of zymosan *in vivo* to activate sequestered neutrophils to induce lung injury. Thus, in the next series of experiments, rolipram or its vehicle was administered after LPS and 30 min before the zymosan. Under these conditions, the vehicle did not significantly alter albumin accumulation in response to LPS plus zymosan treatment (Figure 4a). However, when rolipram was given prior to zymosan, the albumin accumulation was abrogated ($P < 0.01$ compared with LPS and zymosan; Figure 4a) and was not significantly different to saline-treated controls.

As previously seen, LPS plus zymosan treatment significantly increased lung MPO activity when compared with saline-treated controls ($P < 0.01$; Figure 4b). However, in contrast to the effect of rolipram administered 30 min before LPS (*see* Figure 1b), treatment with rolipram after LPS and 30 min before zymosan resulted in a reduction of MPO levels by only 47% ($P < 0.05$); this was found to be significantly ($P < 0.05$) elevated above the MPO levels measured in lung tissue from saline-treated controls.

Figure 2. Histologic appearance of lung tissue from LPS and zymosan-treated mice. (*A*) Lung tissue from a control saline-treated mouse shows normal alveolar structure devoid of inflammatory cell infiltrates or edema formation. (*B*) The alveolar interstitium from an LPS and zymosan-treated animal shows marked sequestration of inflammatory cells which are predominantly neutrophils, identified by their ringed nuclei. (*C*) Pretreatment with rolipram attenuated neutrophil sequestration induced by administration of LPS and zymosan. (*D*) In contrast, rolipram treatment after LPS administration but 30 min before zymosan had no visible effect on neutrophil sequestration. Scale bar in panel = 63 μm.

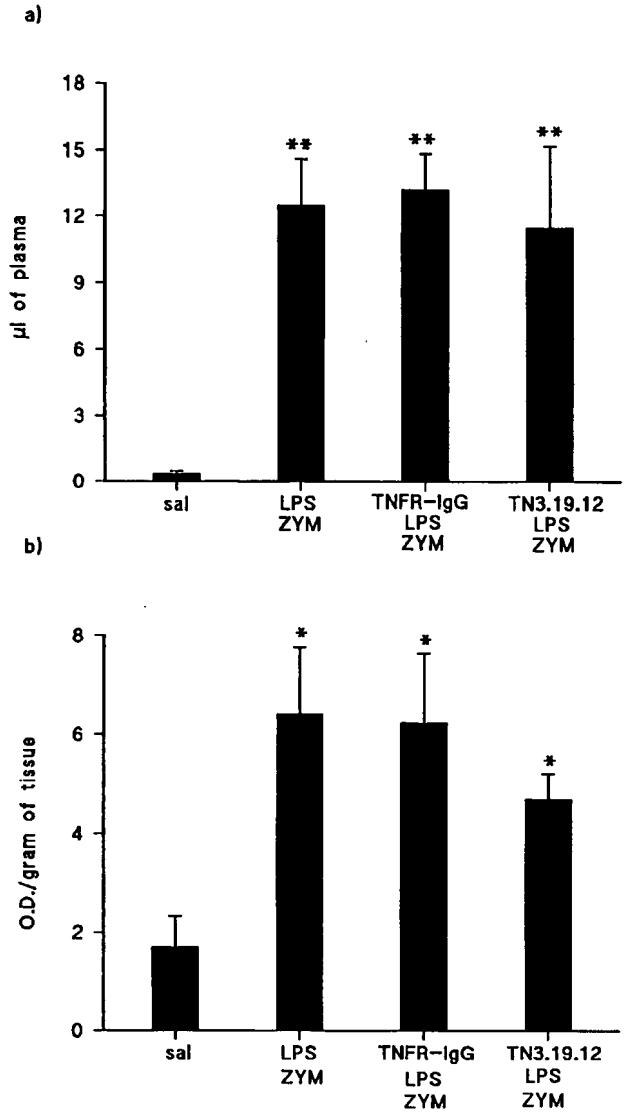

Figure 3. Effect of TNFR-IgG or TN3.19.12 on LPS and zymosan-induced lung injury. The soluble TNFα receptor TNFR-IgG was administered at a dose of 5 mg/kg (i.p.) 6 h prior to LPS and zymosan treatment. Similarly, the mAb TN3.19.12 was administered (30 mg/kg, i.v.) 1 h prior to further treatment. (*a*) Pretreatment with either TNFR-IgG or TN3.19.12 had no significant effect on LPS and zymosan-induced extravascular albumin accumulation. Results are expressed as mean ± SEM of 6 animals where $^{**}P < 0.01$ compared with saline. (*b*) Blockade of TNFα with either TNFR-IgG or TN3.19.12 had no significant effect on MPO levels induced by LPS and zymosan treatment. Results are expressed as mean ± SEM of 3 animals where $^{*}P < 0.01$ compared with saline.

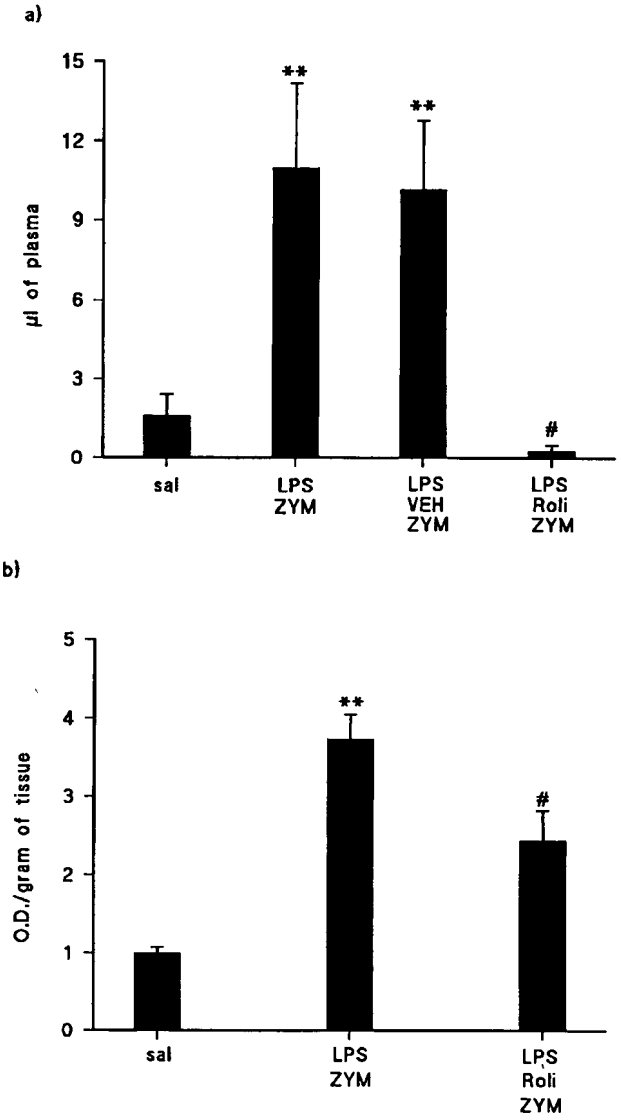

Figure 4. Effect of rolipram on acute lung injury when administered after LPS but before zymosan. (*a*) Rolipram (5 mg/kg, i.p.) given after LPS and 30 min before zymosan significantly reduced albumin accumulation in lung tissue. Results are expressed as mean ± SEM of 3–8 animals where $^{**}P < 0.01$ compared with saline and $^{\#}P < 0.05$ compared with LPS and zymosan. (*b*) Treatment with rolipram after LPS but 30 min before zymosan led to a decrease in MPO levels, which remained significantly elevated above saline. Results are expressed as mean ± SEM of 4 animals where $^{**}P < 0.01$ compared with saline and $^{\#}P < 0.05$ compared with LPS and zymosan or saline.

The histologic profile of lung tissue taken from animals receiving rolipram after LPS and before zymosan confirmed the above data. The extent of neutrophil sequestration appeared to be as marked as in the LPS and zymosan group (*see* Figure 2D).

Effect of Rolipram Pretreatment on Lung Injury Induced by Zymosan Alone

In separate groups of animals, the effect of rolipram was assessed on lung injury induced by zymosan alone. Pretreatment with the vehicle had no significant effect on zymosan-induced albumin accumulation in whole lung tissue, when compared with lung injury induced by zymosan alone

(Figure 5a). However, there was a significant decrease (approximately 86%) in the permeability induced by treatment with zymosan in the animals pretreated with rolipram.

In addition, quantification of neutrophil sequestration in lung tissue, in response to zymosan treatment by assay of MPO activity, indicated a 5-fold increase when compared with saline-treated controls. This is similar to the magnitude of neutrophil sequestration quantified at the electron microscopic level in pulmonary capillaries after zymosan administration (31). In contrast to its inhibitory effect on vascular permeability, rolipram had a partial (approximately 44%) but nonsignificant inhibitory effect on MPO activity (*see* Figure 5b).

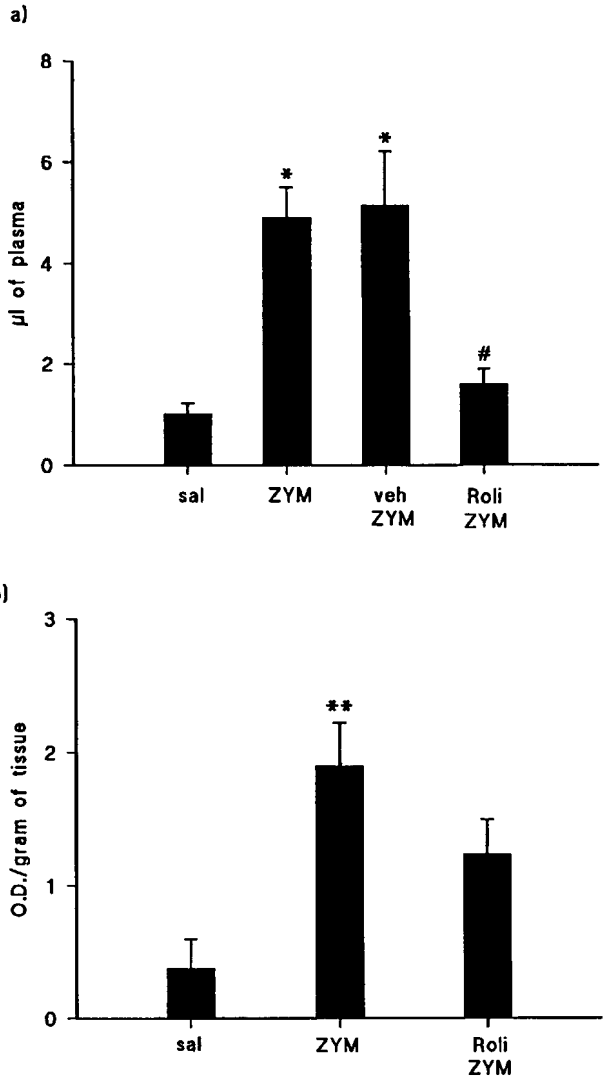

Figure 5. Effect of pretreatment with rolipram on lung injury induced by zymosan alone. (*a*) Pretreatment with rolipram (5 mg/kg, i.p.) 30 min prior to zymosan significantly reduced extravascular albumin accumulation. Results are expressed as mean ± SEM of 3–6 animals where $^{*}P < 0.05$ when compared with saline and $^{\#}P < 0.05$ compared with vehicle plus zymosan. (*b*) Rolipram pretreatment had no significant effect on the MPO levels measured as a result of zymosan treatment. Results are expressed as mean ± SEM of 4 animals where $^{**}P < 0.01$ compared with saline.

Discussion

Attenuation of experimental lung injury has previously been shown to be achieved by treatment with nonspecific inhibitors of PDE. Thus, pentoxifylline leads to a decrease in neutrophil sequestration and vascular permeability increases induced by LPS treatment (29, 40). However, pentoxifylline is a nonspecific and weak PDE inhibitor and may also have other actions. More recently, there have been suggestions that attenuation of TNFα production may be the protective mechanism by which the type 4 PDE inhibitor, rolipram, inhibited LPS-induced lung injury in rats (30). Attenuation of increases in serum TNFα coincided with a decrease in neutrophil accumulation in lung tissue. However, the study did not address the role of endogenous TNFα in the induction of lung injury. Thus the mechanisms by which PDE4 inhibition can inhibit the induction of lung injury and the role of endogenous TNFα in the injury process remain unclear.

It has therefore been the aim of the present study to determine whether a PDE4 inhibitor could also attenuate lung injury in a mouse model, and to assess whether this is related to inhibition of TNFα production. We report that pretreatment with the specific PDE4 inhibitor rolipram before LPS administration leads to an attenuation of neutrophil sequestration in pulmonary capillaries as measured by MPO, an inhibition of lung injury as measured by extravascular albumin accumulation, and a decrease in serum TNFα levels. It has been demonstrated previously that serum TNFα after LPS administration is detected within 30 min and has been shown to peak around 90 min in mice (41). Since production of TNFα occurs early after LPS treatment, TNFα, in addition to LPS, may be involved in the induction of neutrophil sequestration in pulmonary capillaries. TNFα is known to upregulate cell adhesion molecule expression on the leukocytes and endothelial cells (42, 43), and we have previously shown the integrin CD11b and its ligand ICAM-1 to be involved in the sequestration of neutrophils and the induction of injury in this model (31, 44).

It was therefore considered important to investigate the role of endogenous TNFα in mediating LPS-induced lung injury. Neutralization of TNFα by TNFR-IgG in the present study had no significant effect on lung injury. In addition, use of a specific antimouse TNFα antibody also had no effect either on the LPS and zymosan-induced increases in extravascular accumulation of albumin, or on the induced sequestration of neutrophils as measured by the MPO assay. The treatment protocols were exactly as described in studies in which these reagents inhibited LPS-induced mortality (34, 35) and were effective in neutralizing the biologic activity of serum TNFα as assessed by the WEHI assay. Remick and colleagues (45) reported that anti-TNFα antiserum partially reduced LPS-induced neutrophil sequestration in mice, as measured by lung MPO activity, but the study did not measure lung injury. In contrast, Gatti and associates (46) reported that anti-TNFα antibodies did not block pulmonary edema or neutrophil

sequestration induced by LPS in mice, but did attenuate LPS-induced lethality. Thus there appears to be some controversy surrounding the precise role of TNFα in the induction of experimental lung injury. Our data on the mouse are consistent with the study of Pugin and coworkers (47), which reported that the proinflammatory activity in BAL fluid from ARDS patients was due to interleukin-1 and not TNFα.

Since TNFα is not involved in the induction of lung injury, the protective effect of PDE4 inhibition must be attributable to other mechanisms. It is possible, for example, that PDE4 inhibition by treatment with rolipram prior to LPS can inhibit the upregulation of cell adhesion molecules. The induction of lung injury in this present model has previously been demonstrated to be dependent on functional expression of CD11b/18 and ICAM-1 (31, 44). Pober and colleagues (48) have reported that the nonspecific PDE inhibitor isobutyl methylxanthine decreases synthesis and expression of E-selectin and VCAM-1 adhesion molecules in human umbilical vein epithelial cell cultures in response to TNFα; however, there was no effect on ICAM-1 expression. In addition, we have found that rolipram is a poor inhibitor of LPS-induced ICAM-1 expression on human lung microvascular endothelial cells (unpublished observations). In contrast, Derian and associates (49) found that rolipram inhibited N-formyl-methyl-leucyl-phenylalanine-induced upregulation of the β_2 integrins CD11a and CD11b on neutrophils. A similar mechanism may contribute to the capacity of rolipram to impair neutrophil sequestration and the resulting lung injury in the present model.

Neutrophil sequestration in pulmonary capillaries is also induced when cells are rendered less deformable. *In vitro* studies have shown that LPS renders neutrophils less deformable, and this is associated with increased assembly of the F-actin filaments (50). Thus, *in vivo*, the passage of neutrophils through the pulmonary microvasculature and through the lung is likely to be impaired in response to LPS as a result of changes in the neutrophil cytoskeleton. Treatment with rolipram leads to an elevation of intracellular cAMP in circulating leukocytes that may attenuate LPS-induced deformability and prevent the sequestration of neutrophils in pulmonary capillaries. Indeed, cAMP elevating agents render neutrophils more deformable *in vitro* (51), and this may contribute to the significant reduction of neutrophil sequestration in lung vasculature in rolipram-pretreated mice.

We have previously demonstrated that increases in extravascular albumin accumulation were not detected in animals receiving LPS alone (31). Additional activation of the neutrophils by zymosan was required to induce lung injury. The activation process is likely to be independent of endogenously liberated TNFα but associated with systemic complement activation as well as phagocytosis of the zymosan particles (31). Treatment with rolipram after LPS and 30 min before zymosan administration led to a complete inhibition of vascular permeability changes, but there was a less-marked effect on sequestration of neutrophils. In this model, rolipram was administered after the reported peak of TNFα production and so the effect was likely to be a direct one, perhaps downregulating neutrophil activation. In addition, rolipram attenuated extravascular albumin accumulation induced by zymosan alone, without significantly decreasing neutrophil sequestration. In the present model, therefore, inhibition of PDE4 *in vivo* directly attenuates neutrophil activation and the ensuing lung injury. In accordance with reports that PDE4 inhibition reduces neutrophil activation (25–27), we believe that rolipram attenuates the production of neutrophil-derived mediators such as superoxide anions, H_2O_2, and platelet-activating factor (PAF), which are known to increase endothelial permeability (52, 53). Furthermore, we have previously demonstrated that endogenous PAF production is crucial to the induction of increased vascular permeability because a PAF antagonist attenuates the measured injury (39). The finding is an important one because it demonstrates that attenuation of neutrophil activation in the lung can be achieved regardless of the prevailing conditions.

Finally, it has been suggested that part of the anti-inflammatory effects of rolipram *in vivo* may be due to its ability to induce the release of endogenous cortisone (54). We have previously shown that a 2-h pretreatment with the steroid dexamethasone effectively inhibited neutrophil sequestration but only partially inhibited increased extravascular albumin accumulation in mouse lung (55). However, dexamethasone was less effective than rolipram at inhibiting extravascular albumin accumulation induced by LPS and zymosan (55) and failed to alter plasma leakage in the lung induced by zymosan alone (unpublished observations). Therefore, although the release of endogenous cortisone may occur after i.p. administration of rolipram, this release is unlikely to account for the marked inhibitory effects of the drug in this mouse lung injury model.

We have demonstrated herein that inhibition of PDE4 has three main anti-inflammatory effects, i.e., attenuation of TNF production, blockade of neutrophil sequestration in pulmonary capillaries, and inhibition of neutrophil activation. The sequestration of neutrophils in pulmonary capillaries occurs rapidly after the onset of sepsis before the development of ARDS. Since it is unlikely that therapeutic intervention could be timed to target the events in this phase, administration of an agent that inhibits activation of neutrophils already sequestered in lung capillaries would therefore be of interest in the clinical condition. In this context, we have shown that a specific PDE4 inhibitor is beneficial in inhibiting induction of lung injury, even after neutrophil sequestration has occurred, and suggest that this class of agents may have utility in ARDS.

Acknowledgments: The authors thank Mr. T. Ansari for the preparation of lung tissue for light microscopy. The WEHI 164 assay was kindly conducted by Dr. D. Butler at the Kennedy Institute of Rheumatology, London, UK. Rolipram was a gift from Dr. J. Fozard, Sandoz (Basel, Switzerland). The soluble human TNFα receptor (p55)-IgG fusion protein (TNFR-IgG) was a gift from Drs. Scallon and Ghrayeb, Centocor, Inc., Malvern, PA. In addition, the anti-TNFα monoclonal antibody TN3.19.12 was donated by Dr. R. Foulkes, Celltech, Slough, UK. This work has been supported by the Clinical Research Committee, Royal Brompton Hospital; the British Lung Foundation; the National Asthma Campaign; and Sandoz.

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Pulmonary Pharmacology & Therapeutics (2001) **14**, 157–164
doi:10.1006/pupt.2001.0291, available online at http://www.idealibrary.com on IDEAL®

PULMONARY PHARMACOLOGY & THERAPEUTICS

Comparison of PDE 4 Inhibitors, Rolipram and SB 207499 (Ariflo™, in a Rat Model of Pulmonary Neutrophilia

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SUMMARY: Using a rat model of lipopolysaccharide (LPS)-induced pulmonary inflammation, the anti-inflammatory activity of SB 207499 was evaluated and compared to that of the prototypic type-4 phosphodiesterase (PDE4) inhibitor, rolipram. In dose–response experiments, we found that rats exposed to 10 μg or 100 μg of intratracheal (it) LPS developed a prominent pulmonary inflammation, due to a significant increase in the number of recoverable bronchoalveolar lavage neutrophils. The pulmonary neutrophilia, provoked by the challenge of 10 μg LPS/rat, was significant at 2 h, peaked by 16 h, declined thereafter but remained elevated for up to 48 h. Additionally, the exposure of rats to 10 μg LPS caused the local pulmonary production of TNF-α. In contrast to the cellular influx, TNF-α production peaked at 2 h and rapidly declined to negligible levels by 8 h. While low levels were detected, the levels of IL-1β in bronchoalveolar lavage did not significantly differ from saline challenged animals. Rats pretreated with rolipram or SB 207499, displayed dose-dependent inhibition of the LPS-induced pulmonary inflammation. Nevertheless, the pulmonary production of TNF-α and IL-1β was unaffected by either SB 207499 or rolipram. When provoked with the 10 μg dose of LPS, adrenalectomized rats produced a similar 24 h induction of pulmonary neutrophilia. Pretreatment of adrenalectomized rats with the PDE4 inhibitors showed similar inhibitory results to those obtained in normal rats. In summary, we have shown, using a rat model of LPS-induced pulmonary neutrophilic inflammation, that the inhibitory activities of rolipram or SB207499 are not linked to the production of TNF-α or the inhibition of IL-1β, and occur independently of endogenous catecholamine or corticosteroid release.

KEY WORDS: Chronic bronchitis (COPD), SB 207499, Rolipram, LPS, Neutrophilia.

INTRODUCTION

Pulmonary neutrophilia is a consistent finding in human Adult Respiratory Distress Syndrome (ARDS) and in Chronic Obstructive Pulmonary Disease (COPD).[1–3] In these diseases, it is hypothesized that the pulmonary recruitment and activation of neutrophils contribute to the observed dysfunctional symptoms, which include increased airway reactivity, presence of pulmonary fibrosis, and the hypersecretion of mucus.[4,5] Pharmacological agents that decrease both the recruitment and state of activation of pulmonary neutrophils may offer therapeutic advantages by controlling the pathology of these disease entities.[6]

The state of activation of inflammatory cells is influenced by the intracellular levels of cyclic adenosine monophosphate (cAMP), whose level is regulated by the activities of a family of phosphodiesterase isoenzymes (PDE). Through the use of selective isoenzyme inhibitors, it has been documented that the predominant inflammatory cell cAMP-metabolizing enzyme is the type-4 (PDE4).[7–10] Rolipram has been the prototypic compound of this class, but recently SB 207499 (Ariflo™) has been introduced as a more potent and selective PDE4 inhibitor.[11] PDE4 inhibitors are also noted as potent inhibitors of tumor necrosis factor (TNF)-α production.[12,13] Certainly, the production of TNF-α has been documented as one of the mediating factors in the pulmonary recruitment of neutrophils.[14,15]

In these studies, we report on a rat model of lipopolysaccharide (LPS)-induced pulmonary inflammation, characterized by the recruitment of neutrophils, which is partially driven by the local

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production of TNF-α. Using this model of LPS-induced pulmonary neutrophilia, we compared the activity of rolipram and SB 207499 to inhibit the pulmonary inflammation and TNF-α production in normal rats. Furthermore, to address the potential that these inhibitors mediate their primary effect through endogenous corticosteroid or catecholamine release, we examined the antiinflammatory activity of SB207499 and rolipram in LPS-challenged adrenalectomized rats.

METHODS

Male Sprague/Dawley rats (normal and bilateral adrenalectomized; 200–250 g) were purchased from Charles River Laboratories. Prior to use the animals were permitted unrestricted access to food and water. However, in the case of adrenalectomized animals, 0.9% saline was substituted for water. Additionally, after their use, a necropsy was performed on the adrenalectomized animals to ensure that the adrenals were completely removed. All experiments were approved in accordance with NIH guidelines, in a program approved by the American Association for the Accreditation of Laboratory Animal Care.

LPS-treatment

Animals were anesthetized by inhalation of isoflurane, supplemented with oxygen (flow rate 1.0 ml/min). Once anesthetized, animals were placed supine and the trachea visualized using a small laryngoscope. Animals then received either 0.1 ml of saline or 0.1 ml of a 100 μg/ml LPS solution (*Escherichia coli*) in saline by use of a Penn-Century Microspray needle (Penn-Century, Philadelphia, Pennsylvania, USA). Animals were allowed to recover on a heat pad, returned to housing and permitted access to food and water ad libitium. Afterwards, at appropriate time points, animals were anesthetized with an intraperitoneal injection of the combination of ketamine/xylazine (10:1, 200 mg/kg ketamine, 20 mg/kg xylazine). After reaching anesthesia, animals were surgically prepared for bronchial lavage by inserting a tracheal cannula. Animals were lavaged with 2 × 2 ml of phosphate buffered saline, pH 7.2 (PBS). Routine recovery of bronchial lavage (BAL) fluids did not significantly differ between animals with >80% of instilled volume recovered. Afterwards, animals were euthanized by surgically opening the thoracic cavity and cutting the diaphragm to assure lung collapse. Bronchial lavage fluid was analyzed for cytokines and cellular contents as described below.

BAL samples

Bronchial lavage fluid was spun at 350 × *g* for 10 min at 4°C. One ml of supernatant was removed and stored at −20°C until analysed for cytokine levels. Remaining fluid was aspirated and the cell pellet lysed for residual erythrocytes and resuspended in PBS, pH 7.2 containing 10 μg/ml of DNase I. Afterwards, the cell suspension was centrifuged at 350 × *g* for 10 min at 4°C, the supernatant aspirated and the cell pellet resuspended in 1 ml of PBS with 10 μg/ml DNase I and 5% heat-inactivated fetal bovine serum. Cytospin slide preparations were made and stained with Hema3™ staining system (Fisher Scientific, Springfield, New Jersey, USA). Differential cell counts were performed using standard histological parameters and at least 200 cells were enumerated. Total cell counts were performed using a Neubauer chamber.

Rat TNF-α, interleukin (IL)-1, and IL-10 levels were determined using commercial enzyme-linked immunosorbent assay (ELISA) kits from R + D Systems (R + D Systems, Minneapolis, Minnesota, USA). For TNF-α measurements, samples were processed according to the manufacturer's protocol with serum samples being diluted 1:2 and BAL samples diluted 1:3 in diluent buffer. For the assay of IL-1α, IL-1β and IL-10, samples were assayed undiluted. In all cases, samples were assayed in duplicate and the average values calculated off of a standard curve. Absorbance values for samples that fell below the lowest standard value of the assay were assigned the lowest standard values for purposes of statistical evaluation. The limit of detection for TNF-α was 12 pg/ml, 51 pg/ml for IL-1α, 26 pg/ml for IL-1β, and 31 pg/ml for IL-10.

Drug treatment

Animals were fasted overnight prior to administration of rolipram or SB 207499, but were allowed unrestricted access to water. Drugs were suspended in a 0.4% methylcellulose vehicle and given orally 2 h prior to the LPS challenge. All animals tolerated dosing with either the vehicle or drug.

Statistical analysis

Data are presented as mean ± standard error of the mean (SEM) with statistical significance, determined between groups, using an analysis of variance (ANOVA) and a post-hoc analysis of Fisher's protected least square difference (PLSD) on a program from Statview™ (version 4.0, ABACUS Concept, Berkeley, Calicornia, USA).

Reagents

LPS (Lot# 69H4046) and rolipram (Lot# 89H4600) were purchased from Sigma Chemical Co. (St. Louis, Missouri, USA). SB 207499 was synthesized by Richman Chemical Inc (Lot# SK13899, Lower Gwynedd, Pennsylvania, USA) and tested for in vitro potency using cloned PDE4 enzyme (Dr P. Wang, Allergy, SPRI). All other reagents were purchased from standard laboratory suppliers.

RESULTS

LPS-challenge in normal rats

Preliminary experiments showed that, 24 h after the intratracheal (it) injection, LPS elicited a dose-dependent increase in the number of recoverable bronchial lavage (BAL) total cells, compared to saline challenged animals. While no substantial inflammatory response was noted with the 1 μg/rat dose of LPS, at doses of 10 and 100 μg/rat, LPS induced a significant increase in the recoverable BAL total cells (Fig. 1A). This increase in total cells was predominantly due to the recruitment of neutrophilic cells. Twenty-four hours after saline challenge, rats routinely had less than 10% of the recoverable BAL cells being neutrophils (Fig. 1A, B) However, after LPS challenge, with either 10 or 100 μg/rat, the percentage of neutrophils in the BAL rose significantly to 76% and 91%, respectively (Fig. 1A, B). The absolute numbers of mononuclear cells were also significantly elevated after either 10 or 100 ug LPS challenge (Figure 1C). However, compared to saline challenged rats, the percentage of mononuclear cells found in the BAL of the 10 or 100 μg challenged animals declined from 92% to 24% and 9%, respectively. As the 10 μg/rat dose of LPS gave a substantial and significant increase in pulmonary cells, which was predominantly a neutrophilic response, and the animals displayed less physical distress we chose this dose for further evaluation in our studies.

The temporal pulmonary inflammatory response was examined in rats exposed to a single 10 μg/rat dose of LPS. Two hours after LPS-challenge, rats also displayed a significant increase in their recruitment of recoverable BAL pulmonary inflammatory cells, when compared with saline challenged animals (Fig. 2A). Temporally, after The LPS-induced inflammatory response peaks between 8 and 16 h, declines by 24 h, but remains significantly elevated for up to 48 h after challenge (Fig. 2A). Similar to our initial findings, at all time points, the LPS-induced increase in inflammatory cells was primarily due to the recruitment of neutrophils. Following a parallel course neutrophil influx was significant by 2 h, peaked between 8–16 h, declining by 24, but significantly elevated for up to 48 h post-LPS-challenge (Fig. 2B). At no time during the time-course, was a significant influx of inflammatory cells readily observed within the saline treated animals (Fig. 2A, B).

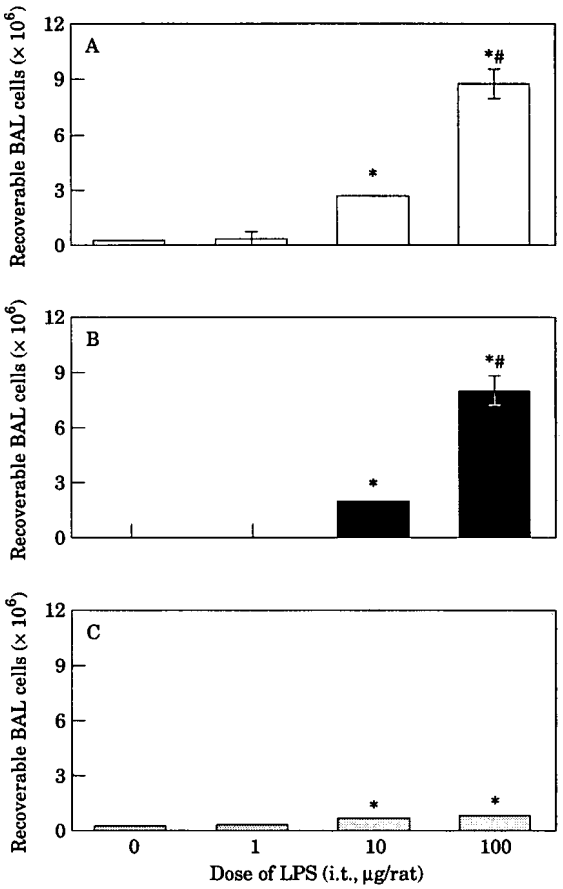

Fig. 1 Dose–response to LPS on the recoverable bronchoalveolar lavage (BAL) cells. Anesthetized rats (n = 4 per group) were exposed to a single intratracheal challenge of saline, 1, 10 or 100 μg of LPS (*E. coli*) in a 0.1 ml aliquot. Twenty-four hours later animals were euthanized and BAL performed using 0.9% saline, according to the Methods. Total cells (A) counts were performed using Neubauer chamber and differentials, neutrophils (B) and mononuclear cells (C), enumerated using normal histological parameters on Leukostat™-stained Cytospin preparations. Values reported are means ± SEM. Statistical significance of (*) $P<0.05$ compared to saline and (#) $P<0.05$ compared to 10 ug dose.

In vivo cytokine production

The production of TNF-α was evaluated by testing the serum and BAL fluids from saline and LPS-challenged rats. The analysis of BAL fluids from saline-treated animals generally contained less than the lowest value of the ELISA standard curve of 12 pg/ml of TNF-α. Furthermore, the BAL of saline-treated animals often had no observable neutrophils.

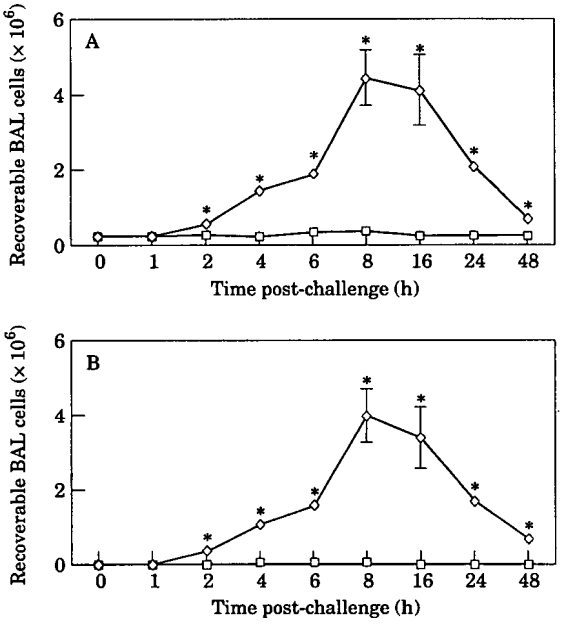

Fig. 2 Time-course of LPS-induced pulmonary inflammation in rats. Anesthetized animals (n=4 per group/time point) were exposed to a single intratracheal administration of saline (—□—) or 10 μg/rat of LPS (*E. coli*; —◇—) in a 0.1 ml aliquot. At the appropriate times after challenge, animals were anesthetized and BAL performed according to the Methods. Cell suspensions were processed for total cells (A) and neutrophilic cells (B) as described in Fig. 1 and the Methods. Values are reported as means ± SEM, with significance of (*) $P<0.05$ compared to saline.

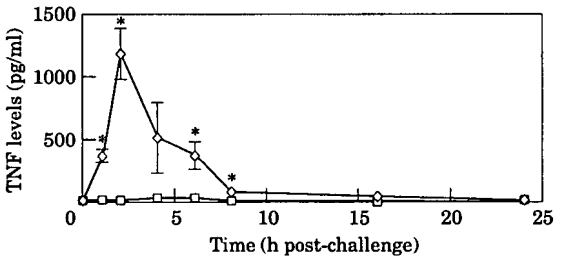

Fig. 3 Time-course of TNF-α production in BAL from LPS-challenged rats. BAL fluids were recovered from rats exposed to a single 10 μg dose of LPS, detailed in Fig. 2; n=4 per group/time point. BAL fluids were analyzed for TNF-α levels using a commercial ELISA for RAT TNF-α according to manufacturers' protocol (see Methods). The lower limit of detection for the assay was 12 pg/ml. Values reported are means ± SEM with statistical significance determined as (*) $P<0.05$ compared to saline. Saline, —□—; LPS, —◇—.

In contrast, BAL fluids from LPS-challenged rats (10 μg/rat) contained measurable levels of TNF-α. Differing from the peak neutrophilic inflammatory response at 8–16 h, the peak in TNF-α production occurred 2 h after LPS-challenge (Fig. 3, 1186 ± 204 pg/ml, $P<0.05$ compared to saline). Declining thereafter, TNF-α levels returned to baseline values by 16 h. No significant levels of TNF-α were found at any time in the serum of saline-or LPS-challenged animals (data not shown).

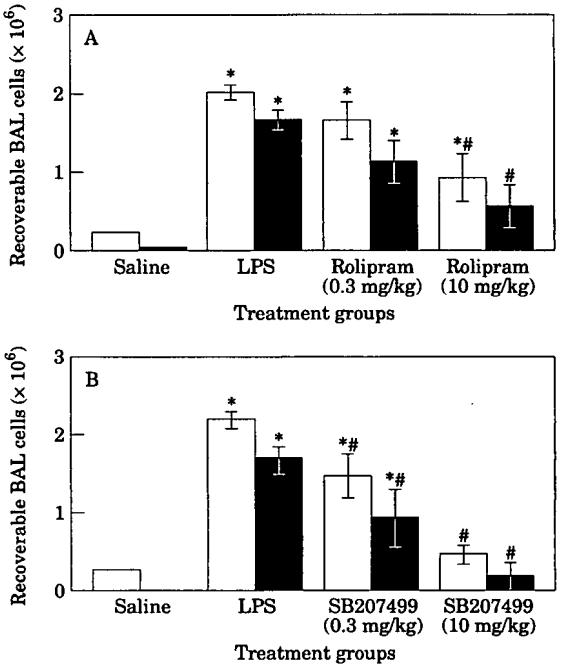

Fig. 4 Effect of PDE4 inhibitors, rolipram and SB 207499, on recoverable BAL cells from LPS-challenged rats. Rats (n=5 per group) were pretreated, orally with vehicle, rolipram (A) or SB 207499 (B; 0.3 or 10 mg/kg), 2 h prior to a single challenge with 10 μg of LPS. Twenty-four hours later animals were anesthetized and BAL performed. Total cells and differentials were performed as described in the Methods. Values reported are means ± SEM. Statistical significance was determined as (*) $P<0.05$ compared to saline and (#) $P<0.05$ compared to LPS. Total cells, □; neutrophils, ■.

At 2 h, the BAL from saline challenged animals contained low but detectable levels of IL-1β, 40 ± 3 pg/ml. However, in contrast to TNF-α levels, the BAL from LPS-challenged rats was not significantly different from the BAL from saline-challenged animals, 60 ± 15 pg/ml. The levels for IL-10 and IL-1α, in BAL samples of saline or LPS challenged rats, were always below the limit of detection of the assay (data not shown).

Antiinflammatory effect of rolipram or SB 207499

Compared to LPS challenged animals, rats pretreated with rolipram or SB 207499 showed a dose-dependent decrease in the number of recoverable inflammatory BAL cells. Rolipram given at 0.3 mg/kg, p.o., 2 h prior to LPS challenge was able to affect a modest, but not statistically significant, inhibition of the LPS induced pulmonary inflammation by blocking total cell influx by 20% and neutrophil influx by 33% (Fig. 4A). When given orally at 10 mg/kg, rolipram effected a dramatic

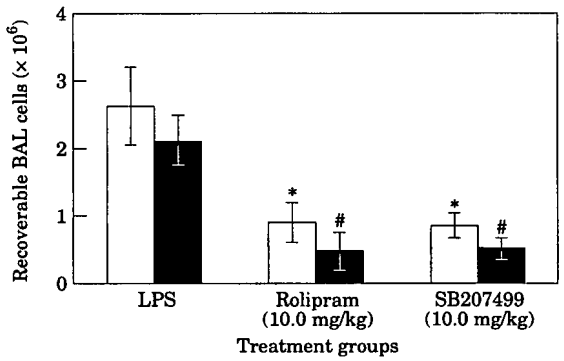

Fig. 5 Effect of rolipram and SB207499 on LPS-induced inflammation in adrenalectomized rats. Rats (n = 5 per group) were pretreated, orally with vehicle, rolipram or SB 207499 (10 mg/kg) prior to a single challenge with 10 μg of LPS. Twenty-four hours later animals were anesthetized and BAL performed. Total cells and differentials were performed as described in the Methods. Values reported are means ± SEM. Statistical significance was determined as (*) $P<0.05$ compared to LPS-total cells and (#) $P<0.05$ compared to LPS-neutrophils.

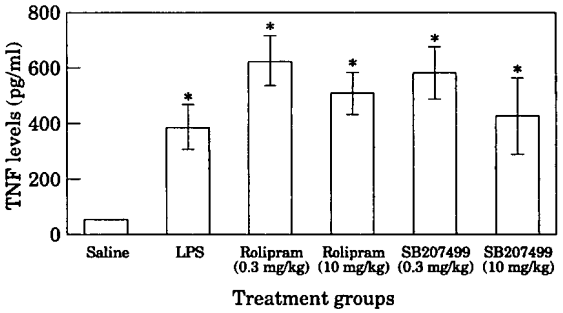

Fig. 6 Effect of rolipram or SB 207499 on LPS-induced pulmonary production of TNF-α in rats. Rats (n = 7) were orally pretreated (2 h prior to challenge) with vehicle, rolipram or SB 207499 (0.3 or 10 mg/kg) and then challenged with a single dose of 10 μg/rat of LPS. Animals were anesthetized and BAL performed according to the Methods. BAL fluids were analyzed as for TNF-α levels as described in Fig. 3 and the Methods. Values reported are means ± SEM. Statistical significance was determined as (*) $P<0.05$ compared to saline.

and statistically significant decrease in total cells by 62% and neutrophils by 67% (Fig. 4A).

At equal doses, SB 207499 was more effective than rolipram in suppression of the LPS-induced pulmonary inflammation. SB 207499 at 0.3 mg/kg, p.o., 2 h prior to LPS challenge effected a significant 38% inhibition of total cells and 47% inhibition of neutrophils (Fig. 4B). When given at 10 mg/kg, SB 207499 abolished the pulmonary inflammation by causing a 90% reduction in both the total cells and neutrophil influx due to LPS challenge (Fig. 4B). The BAL profile from animals pretreated with SB 207499 at 10 mg/kg was not significantly different from saline challenged animals ($P>0.05$).

LPS-induced pulmonary inflammation in adrenalectomized rats

Similar to normal rats, adrenalectomized rats exposed to an intratracheal challenge of LPS (10 μg/rat) developed a significant pulmonary inflammation after 24 h, characterized by a predominance of neutrophils (Fig. 5). Adrenalectomized rats pretreated (2 h prior to LPS challenge) with rolipram or SB 207499 (10 mg/kg) displayed a significant inhibition of the 24 h, LPS-induced total cell recruitment and neutrophilia (Fig. 5). In the interest of animal care and use, saline challenged, adrenalectomized animals were not examined.

In vivo anti-TNF-α activity of rolipram or SB 207499

As the peak levels of TNF-α occur at 2 h after LPS challenge, we examined the BAL fluids from animals pretreated with rolipram or SB 207499, at this time after LPS challenge. Neither rolipram nor SB 207499 affected a suppression of BAL levels of TNF-α after LPS challenge (Fig. 6A). To assure that compounds were appropriately administered, we also examined their effect on the LPS-induced cellular influx at this time point. There was a significant increase in BAL recoverable total cells (saline $= 0.27 \times 10^6 \pm 0.03 \times 10^6$; LPS $= 0.59 \times 10^6 \pm 0.03 \times 10^6$ cells; $P<0.05$) and neutrophils (saline $= 0.03 \times 10 \pm 0.03 \times 10^6$; LPS $= 0.39 \times 10 \pm 0.03 \times 10^6$ cells; $P<0.05$) 2 h after LPS challenge, which was inhibited by both rolipram and SB 207499 at 10 mg/kg. Rolipram at 10 mg/kg, blocked total cell influx ($0.46 \times 10^6 \pm 0.05 \times 10^6$) by 42% and neutrophils ($0.20 \times 10^6 \pm 0.05 \times 10^6$) by 52%, while SB207499 at 10 mg/kg blocked total cell influx ($0.34 \times 10^6 \pm 0.04 \times 10^6$) by 79% and neutrophil influx ($0.1 \times 10^6 \pm 0.04 \times 10^6$) by 78%. However, at 2 h post-LPS-challenge neither rolipram nor SB 207499 at 0.3 mg/kg was effective at inhibiting total cells or neutrophils.

We also detected BAL levels of IL-1β from the saline (40 ± 3 pg/ml) and LPS-challenged (60 ± 15 pg/ml) rats, which was not significantly different between the treatment groups. At 2 h post-LPS-challenge we assayed for IL-1β levels, in BAL samples, from the rolipram-and SB 207499-pretreated animals. Neither drug altered BAL IL-1β levels at this time point (data not shown). The effect of rolipram or SB 207499 for the stimulation of IL-10 production after LPS challenge was also examined at 2 h, and no measurable levels of IL-10 from either drug-or vehicle-treated rats could be seen.

DISCUSSION

The presence of activated neutrophils has been postulated to be a detrimental component in at least two

human pulmonary disease conditions, namely, Adult Respiratory Disease Syndrome (ARDS) and Chronic Obstructive Pulmonary Disease (COPD). In both cases, the presence of pulmonary neutrophils and their extracellular deposition of granular products is associated with an increase in airway dysfunction, mucus hypersecretion, tissue destruction and airway remodeling.[1,4] Similarly in both disease entities, the increased exposure to bacterial byproducts, e.g. endotoxin, has been suggested as a partial explanation for the recruitment of neutrophils.[16-18]

Numerous investigators have used LPS to induce inflammation in rodent models of human disease.[19-22] We developed our model to confirm the utility of LPS to induce pulmonary inflammation in rats, as measured by cellular influx and to examine the potential use of PDE4 inhibitors to attenuate this pulmonary inflammation. First, we found that we could establish an inflammatory dose–response relationship to the intratracheal administration of LPS in rats. At the highest dose of LPS (100 μg/rat), numerous animals displayed respiratory distress which included rales and an overall profile of physical discomfort. While no animals died at the higher dose, we chose to use a 10 μg LPS challenge/rat paradigm to conduct our studies because we could establish a significant pulmonary inflammation while minimizing the stress to the animals.

In our model, within 2 h after LPS challenge (10 μg/rat) a substantial increase in pulmonary inflammatory cells, represented predominantly by neutrophils, occurs. This pulmonary neutrophilia remained significantly elevated for 48 h after a single exposure to LPS. Additionally, we found that 2 h after a single challenge with LPS there is a significant increase in BAL levels of TNF-α. We propose that the production of TNF-α in this model is a local response. This conclusion is based on our findings showing that while dramatic increases in BAL levels of TNF-α were found after LPS challenge, we were unable to detect measurable levels of TNF-α in serum samples. This is similar to the results from Miller-Larsson et al, who documented a single rise in BAL TNF-α levels at 2 h in samples from normal rats exposed to (it) LPS.[23]

Interestingly, in contrast to another model of LPS challenge, our model of LPS-induced pulmonary neutrophilia did not result in a significant production of IL-1.[19,24] After a single 10 μg, (it) LPS-challenge, we were unable to find measurable levels of IL-1α or significant alterations in the production of IL-1β. Therefore, we believe our model of pulmonary neutrophilia is not dependent on the presence or differential production of IL-1. The mechanism by which our LPS-induced neutrophilia is separated from IL-1 production may be due to our use of a substantially lower LPS challenge. In most other models, the LPS challenge is significantly higher, often by the order of ten-fold.[19,23,24]

Due to the temporal peaks associated with the cytokine and cellular components, we chose to look at the inhibitory activity of the PDE4 compounds, rolipram and SB 207499, at 2 and 24 h after the LPS challenge. Despite the differences between animal species used, our results confirm previous reports for antiinflammatory activity for rolipram and SB 207499 in reducing pulmonary inflammation provoked by LPS-challenge.[25,26] We have added to those earlier results by incorporating a comparison between rolipram and SB 207499 in the same animal system. In the experiments reported here, we found that SB 207499 appears to be more potent than rolipram in blocking pulmonary neutrophilia, induced by a single LPS challenge in rats. Additionally for the first time, we have documented a dose-dependent inhibition of LPS-induced pulmonary inflammation by SB 207499.

Other investigators have shown that part of the mechanism of action from PDE4 inhibitors is the direct inhibition of TNF-α production and suggest an additional indirect inhibitory mechanism through the reciprocal production of IL-10.[27-30] However, when we examined peak BAL TNF-α levels, at 2 h, neither SB 207499 nor rolipram were effective in decreasing TNF-α levels. This was despite their capacity to block cell influx at this time point. It is interesting to speculate that the PDE4 inhibitors may have altered the temporal expression of TNF-α. However, even a differential time-course for TNF-α production would not explain our observed inhibitory action for the PDE4 compounds on inflammation. Furthermore, the low levels of BAL IL-1β found were not altered by the PDE4 compounds.

As this paradigm of inflammation spontaneously resolves after 48 h, post-LPS-challenge, we investigated the potential that IL-10 is produced somewhere during our time-course. While our model confirms increases in the production of TNF-α after LPS challenge, we were unable to detect production of IL-10. Our inability to detect IL-10 in BAL samples from LPS challenged animals suggests that IL-10 production is not essential for the normal resolution of the inflammatory response. Furthermore, the lack of measurable levels of IL-10 from animals pretreated with either SB 207499 or rolipram, at 2 h post-LPS-challenge, suggests that the production of IL-10 is not an obligatory mechanism for the inhibitory in vivo activities of the PDE4 compounds. Our results support those of Seldon et al, who reported that IL-10 does not play a role in the inhibition of LPS-induced TNF-α production from human monocytes.[31] While we did not examine later time points for IL-10 production, we believe that because inhibitory activity of SB 207499 and rolipram on cell influx could be observed

at 2 h if IL-10 production is important, then measurable amounts of IL-10 would have been expected in the BAL samples.

Nevertheless, several other mediators (e.g. LTB4, CINC or MIP2) may also be involved in the overall inflammatory response due to LPS challenge.[20,32,33] Currently, we are unaware of experimental evidence that documents the utility of PDE4 inhibitors to interrupt the action of these agents. Nevertheless, we have begun experiments to investigate the potential of these agents and their inhibition by rolipram and SB 207499.

Recent attention has been focused on the interaction of PDE4 inhibitors and stimulation of the adrenals.[34–36] As we were successful in using rolipram and SB 207499 to block the inflammatory cell response in adrenalectomized animals, we do not believe that the major antiinflammatory activity of these compounds lies with the endogenous release of catecholamines or corticosteriods. While we cannot rule out a contribution from the adrenals at lower doses of the PDE4 inhibitors, at the highest dose tested (10 mg/kg) both rolipram and SB 207499 were effective at inhibiting LPS-induced inflammation. Assuming the lack of adrenal activity, it is worthwhile to speculate that part of their inhibitory activity lies with a down-regulation in the state of neutrophil activation and adhesion.[37]

In summary, the present study presents the first direct comparison between the PDE4 inhibitors, rolipram and SB 207499, and their ability to attenuate pulmonary neutrophilia. With the recent success of SB 207499 use in the treatment of airway dysfunction of COPD patients, our results are consistent with the overall hypothesis demonstrating the utility of PDE4 inhibitors in the management of this disorder.[38] We found that both compounds were effective at inhibiting LPS-induced pulmonary neutrophilia, but the inhibitory activity was not associated with TNF-α or IL-1 inhibition. Furthermore, the inhibitory activity of these compounds was not associated with either IL-10 production or the endogenous release catecholamines or corticosteriods. Overall, our results continue to support the premise that the use of PDE4 inhibitors may be useful in the control of neutrophilic inflammation in respiratory diseases such as COPD and ARDS.

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Date received: 27 July 2000.
Date revised: 26 January 2001.
Date accepted: 30 January 2001.

0022-3565/05/3142-846–854$20.00
THE JOURNAL OF PHARMACOLOGY AND EXPERIMENTAL THERAPEUTICS
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JPET 314:846–854, 2005

Vol. 314, No. 2
83519/3042847
Printed in U.S.A.

Dynamic Activation of Cystic Fibrosis Transmembrane Conductance Regulator by Type 3 and Type 4D Phosphodiesterase Inhibitors

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Received January 11, 2005; accepted April 29, 2005

ABSTRACT

The diseases of cystic fibrosis, chronic obstructive pulmonary disease (COPD), and chronic bronchitis are characterized by mucus-congested and inflamed airways. Anti-inflammatory agents that can simultaneously restore or enhance mucociliary clearance through cystic fibrosis transmembrane conductance regulator (CFTR) activation may represent new therapeutics in their treatment. Herein, we report the activation of CFTR-mediated chloride secretion by phosphodiesterase (PDE) 4 inhibitors in T84 monolayer using ^{125}I anion as tracer. In the absence of forskolin, the iodide secretion was insensitive to PDE4 inhibitor L-826,141 [4-[2-(3,4-bis-difluoromethoxyphenyl)-2-[4-(1,1,1,3,3,3-hexafluoro-2-hydroxypropan-2-yl)phenyl]-ethyl]-3-methylpyridine-1-oxide], roflumilast, or to PDE3 inhibitor trequinsin. However, these inhibitors potently augmented iodide secretion after forskolin stimulation, with efficacy coupled to the activation states of adenylyl cyclase. The iodide secretion from PDE3 or PDE4 inhibition was characterized at first by a prolonged efflux duration, followed by progressively elevated peak efflux rates at higher inhibitor concentrations. Paralleled with an increased phosphor-cAMP response element-binding protein formation, the CFTR activation dissociated from a global cAMP elevation and was blocked by H89 [*N*-[2-((*p*-bromocinnamyl)amino)ethyl]-5-isoquinolinesulfonamide]. 2-(4-Fluorophenoxy)-*N*-[(1*S*)-1-(4-methoxyphenyl)ethyl]nicotinamide, a stereoselective PDE4D inhibitor, augmented iodide efflux more efficiently than its less potent (*R*)-isomer. The peak efflux from maximal PDE4 and PDE3 inhibition matched that from full adenylyl cyclase activation. These data suggest that PDE3 and PDE4 (mainly PDE4D) form the major cAMP diffusion barrier in T84 cells to ensure a compartmentalized CFTR signaling. Together with their potent anti-inflammatory properties, the potentially enhanced airway mucociliary clearance from CFTR activation may have contributed to the efficacy of PDE4 inhibitors in COPD and asthmatic patients. PDE4 inhibitors may represent new opportunities to combat cystic fibrosis and other respiratory diseases in future.

Respiratory epithelia continuously remove inhaled particles, microbes, and metabolites of resident and migratory cells through a mucociliary clearance process that requires the secretion of fluid and electrolytes. Cystic fibrosis transmembrane conductance regulator (CFTR) is the primary cAMP-activated chloride channel on the apical membrane of airway epithelia, thereby playing an integral role in controlling the electrolyte/fluid balance and mucociliary clearance process (Pilewski and Frizzell, 1999). Most CFTR mutations lead to either a reduced expression or proteins with a compromised chloride conductance in response to physiological stimuli. The major disease mutation ΔF508-CFTR with a decreased epithelial expression in CF patients (Kalin et al., 1999) remains partially functional with a reduced open probability and sensitivity to stimulation by cAMP agonists (Wang et al., 2000a). The functional defects of CFTR mutants result in multiple organ dysfunctions, including a severely impaired airway mucociliary clearance that leads to chronic

Article, publication date, and citation information can be found at http://jpet.aspetjournals.org.
doi:10.1124/jpet.105.083519.

ABBREVIATIONS: CFTR, cystic fibrosis transmembrane conductance regulator; CF, cystic fibrosis; PKA, protein kinase A; AC, adenylyl cyclase; PDE, 3′,5′-cyclic nucleotide phosphodiesterase; COPD, chronic obstructive pulmonary disease; RP-73401, 3-(cyclopentyloxyl)-*N*-(3,5-dichloropyridin-4-yl)-4-methoxybenzamidine; Cpd-A (L-826,141), active enantiomer of 4-[2-(3,4-bis-difluoromethoxyphenyl)-2-[4-(1,1,1,3,3,3-hexafluoro-2-hydroxypropan-2-yl)phenyl]-ethyl]-3-methylpyridine-1-oxide; Cpd-B, 2-(4-fluorophenoxy)-*N*-[(1*S*)-1-(4-methoxyphenyl)ethyl]nicotinamide; Cpd-C, 2-(4-fluorophenoxy)-*N*-[(1*R*)-1-(4-methoxyphenyl)ethyl]nicotinamide; H89, *N*-[2-((*p*-bromocinnamyl)amino)ethyl]-5-isoquinolinesulfonamide; DMSO, dimethyl sulfoxide; RS25344, 8-aza-1-(3-nitrophenyl)-3-(4-pyridylmethyl)-2,4-quinazolinedione; RT, room temperature; TTBS, 20 mM Tris-HCl, pH 7.5, 50 mM NaCl, and 0.1% (v/v) Tween 20; pCREB, phosphor-cAMP response element-binding protein; CREB, cAMP response element-binding protein.

secondary bacteria infections and respiratory failure in CF patients.

It is being increasingly recognized that there is an elevated inflammation in CF patients, which plays a major role in the pathogenesis of CF lung disease (Chmiel et al., 2002). Early pulmonary inflammation with neutrophilia can be detected in CF infants before the onset of bacterial colonization (Khan et al., 1995). Leukotriene B_4 is elevated in the epithelial lining fluid of CF patients (Konstan et al., 1993). CF tissues have abnormally high levels of proinflammatory arachidonic acid with values of the heterozygous parents halfway between CF patients and healthy controls (Freedman et al., 2004). These data suggest the possible presence of an excessive eicosanoid-based inflammatory response in CF patients and CF carriers. Diseases associated with CFTR mutations continue to widen. In addition to the classic CF, approximately 3.3% of the U.S. population are CF carriers who are predisposed to a number of related airway diseases, including chronic bronchitis and rhinosinusitis (Kostuch et al., 2000; Wang et al., 2000b).

The key regulatory event for CFTR activation is the PKA-mediated serine phosphorylations of its R-domain, which facilitates its ATP binding, with the sequential ATP hydrolysis triggering the channel opening and closing. CFTR is dynamically regulated by the activities of adenylyl cyclase (AC) and cAMP-phosphodiesterase (PDE) through the activation of PKA. Multiple signaling elements, including receptors, G proteins, AC, PKA, CFTR, and protein phosphatase 2C cluster at the apical membrane of epithelial cells to ensure the specificity of the cAMP-mediated CFTR activation (Zhu et al., 1999; Sun et al., 2000; Huang et al., 2001a). cAMP hydrolysis in airway epithelial cells is mainly regulated through PDE4s and PDE3s (Wright et al., 1998). There are four PDE4 (4A to 4D) and two PDE3 (3A and 3B) genes, each producing multiple spliced variants with specific tissue distribution and subcellular localization. PDE4s are abundantly expressed in proinflammatory cells and airway epithelial cells. PDE4 inhibition attenuates the overproduction of many proinflammatory mediators and cytokines, including arachidonic acid, leukotrienes, reactive oxygen species, and tumor necrosis factor-α, and suppresses the infiltration of neutrophils and eosinophils in inflamed airways (Torphy, 1998). PDE4D, the major cAMP-PDE species in bronchial epithelia, plays a dominant role in controlling airway smooth muscle contraction (Mehats et al., 2003). A number of second-generation PDE4 inhibitors, exemplified by roflumilast [3-(cyclopropyl-methoxy)-*N*-(3,5-dichloropyridin-4-yl)-4-difluoromethoxy benzamide], are presently in advanced clinical development and may emerge as new therapeutics for asthma and COPD (Huang et al., 2001b).

Restoring CFTR activity and simultaneously reducing the excessive airway inflammatory response may represent a promising strategy in CF treatment. In addition, enhancing the mucociliary clearance via CFTR activation may reduce the respiratory disorders among CF carriers and improve the lung function of COPD patients. This partly stems from the observation that 1) levels of the residual CFTR activity seem to be predictive of the CF disease severity; and 2) cAMP-elevating agents, either through receptor stimulation, AC activation, or nonselective PDE or PDE3 inhibition, activated the wild type-CFTR and partially restored the defective chloride conductance of many CFTR mutants, including ΔF508-CFTR from CF mice and patients (Haws et al., 1996; Kelley et al., 1997; Al Nakkash and Hwang, 1999). Effects of PDE4 inhibitors on CFTR activity had been controversial. Rolipram [4-[3-(cyclopentyloxy)-4-methoxyphenyl]-2-pyrolidinone] and RP-73401 were either inactive or weakly modulated the chloride conductance of Calu3 and T84 cells under conditions where PDE3 inhibition potently increased the short-circuit current measurement (Kelley et al., 1995; O'Grady et al., 2002; Cobb et al., 2003), whereas RS25344 significantly augmented the adenosine-induced apical anion conductance in Calu3 cells (Barnes et al., 2005). By directly monitoring the chloride secretion using ^{125}I as tracer in T84 monolayer, we report here that PDE4 inhibitors, particularly the PDE4D-selective inhibitors, potently augmented CFTR-mediated iodide secretion with their efficacy coupled to the AC activation state.

Materials and Methods

Chemicals. Buffer chemicals were from Sigma-Aldrich (St. Louis, MO). [^{3}H]Cyclic adenosine 3′,5′-monophosphate was from Amersham Biosciences, Inc. (Piscataway, NJ). Complete protease inhibitor tablet was from Roche Diagnostics (Indianapolis, IN). PDE4 inhibitors L-826,141 (Cpd-A), the comparator roflumilast, 2-(4-fluorophenoxy)-*N*-[(1*S*)-1-(4-methoxyphenyl)ethyl]nicotinamide (Cpd-B) and its (*R*)-enantiomer (Cpd-C) were prepared according to literature (Marfat and Chambers, 1998; Reid, 2002; Claveau et al., 2004). Forskolin, H89, and trequinsin [9,10-dimethoxy-2-mesitylimino-3-methyl-2,3,6,7-tetrahydro-(6,1-*a*)-isoquinolin-4-one, HCl] were from Calbiochem (San Diego, CA). Sodium iodide (^{125}I) with a typical specific activity of 2000 Ci/mmol was from Draximage Inc. (Kirkland, QC, Canada).

Cell Culture. T84 cells (American Type Culture Collection, Manassas, VA) were grown as a monolayer in a 1:1 mixture of Dulbecco's modified Eagle's medium/F-12 supplemented with 5% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin. The epithelial cells (1×10^6 cells/well) were plated onto the 9.5-cm^2 culture dishes and incubated at 37°C in a humidified atmosphere of 5% CO_2 in air. Culture medium was changed every 48 h. It becomes confluent in 4 to 5 days. Only cell viability exceeded 90% by trypan blue exclusion was used for further experiments.

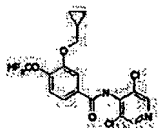
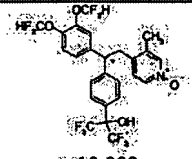
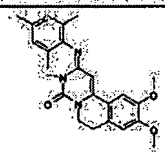
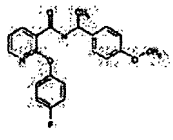
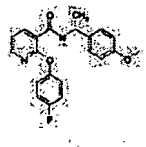
^{125}I Efflux Assay. Confluent T84 monolayer in six-well dishes with a density of 4×10^6 cells/well was labeled with 5 μCi/ml $Na^{125}I$ in 1 ml of HPBR buffer (135 mM NaCl, 5 mM KCl, 3.33 mM NaH_2PO_4, 0.83 mM Na_2HPO_4, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM glucose, and 5 mM HEPES, pH 7.2) for 30 min at 37°C. Labeled cells were washed with 3×5 ml of HPBR buffer within 30 s to completely remove extracellular radioactivity. Efflux of the intracellular ^{125}I was sampled by replacing 80% (0.8 ml) of the incubation buffer with fresh buffer every 30 s up to 9 min. Activators were added via the assay buffer immediately after the third buffer replacement at 1.5 min. Temperature was maintained at 37°C. NaOH (1 ml, 0.1 N) was added after 9 min to lyse the cells by sonication (2 min, Branson model 2510 bath sonicator). The radioactivity in each time point and the final cell lysate were determined on a Microbeta liquid scintillation counter in 24-well format. The maximal forskolin-induced efflux rate over the DMSO control typically ranged from 0.35 to 0.45 min^{-1} for cells up to 10 passages. Studies investigating multiple conditions were paired using cells of same passage.

cAMP Measurement. Confluent T84 cells in 96-well plate were incubated with drug in 200 μl of efflux assay buffer at RT. After quenched with 20 μl of 1 N HCl, cells were lysed by sonication for 2 min. The acidic lysate (20 μl) was neutralized with 80 μl of 250 mM Tris-HCl, pH 7.5, and its cAMP levels were quantified using the cAMP-Biotrak SPA screening assay kit (Amersham Biosciences, Inc.) per manufacturer's directions.

TABLE 1

Potency and selectivity of inhibitors against cAMP-PDEs

IC_{50} values represent mean ± S.D. (in nanomolar), with $n \geq 3$. The potency of roflumilast and Cpd-A have recently been disclosed (Claveau et al., 2004).

Compound	Roflumilast	Cpd-A (L-826,141)	Trequinsin	Cpd-B (S)-isomer	Cpd-C (R)-isomer
Structure					
PDE1	>10,000	>10,000	7800 (1500)	4200 (700)	5300 (1000)
PDE3A	>10,000	2100 (400)	0.04 (0.02)	>10,000	>10,000
PDE3B	>10,000	1100 (100)	0.05 (0.01)	>10,000	>10,000
PDE4A	0.2 (0.05)	1.3 (0.2)	360 (240)	315 (40)	156 (48)
PDE4B	0.1 (0.02)	0.4 (0.3)	437 (490)	200 (50)	76 (26)
PDE4C	0.6 (0.1)	2.4 (1.8)	790 (630)	970 (140)	1160 (450)
PDE4D	0.1 (0.03)	0.3 (0.2)	230 (180)	1.4 (0.3)	39 (10)
PDE7A,	>10,000	>10,000	9000 (1500)	>10,000	>10,000
PDE8B	>10,000	7200 (400)	2100 (500)	>10,000	>10,000

PDE Activity Assay. Confluent T84 cells (~100 × 10^6) were harvested by trypsinization and washed twice using ice-cold phosphate-buffered saline by centrifugation. Cells were suspended in 1 ml of a buffer containing 50 mM HEPES, pH 7.5, 5 mM EDTA, 0.05 mM dithiothreitol, 1 mM *N*-α-benzoyl-L-arginine ethyl ester, 1 mM benzamide, 10 μg/ml pepstatin A, and Complete EDTA-free proteases inhibitors tablet. After sonication (5 × 10-s pulses; power setting 3; 4°C), the lysate was collected after a 10,000*g* spin (30 min at 4°C). The lysate cAMP-PDE activity was determined by monitoring the hydrolysis of 0.1 μM [^{3}H]cAMP in 10 mM $MgCl_2$ and 50 mM HEPES, pH 7.2, using the SPA-PDE assay kit from Amersham Biosciences, Inc. (Laliberte et al., 2000). The potencies of inhibitors against multiple PDEs in Table 1 were determined using 0.1 μM [^{3}H]cAMP for PDE1, PDE4s, PDE7A, and PDE8B and 0.01 μM [^{3}H]cAMP for PDE3A and PDE3B under the same assay conditions as detailed previously (Claveau et al., 2004). Under the conditions, they were close to the apparent K_i. PDE1 was purified from dog heart, and other PDEs were human recombinant enzymes.

Phosphor-CREB Induction and Western Blot Analysis. Confluent T84 monolayer in six-well plate at 37°C was incubated with compounds or DMSO in efflux buffer for specified time. After the removal of incubation buffer, 300 μl of ice-cold lysis buffer [50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM $Na_4P_2O_7$, 0.1 mM vanadate, 250 μM H_2O_2 plus protease inhibitor (EDTA-free) cocktail tablet] was added. Cells were lysed in the bath sonicator (2 min/4°C). Supernatant was collected after a 10,000*g* centrifugation (10 min/4°C) and concentrated by Speed-Vac. Approximately 100 μg of protein was loaded per lane on SDS-polyacrylamide gel electrophoresis under denaturing condition. After electrophoresis, proteins were transferred to polyvinylidene difluoride membranes, and blocked with 5% skim milk in TTBS [20 mM Tris-HCl, pH 7.5, 50 mM NaCl, and 0.1% (v/v) Tween 20] for >60 min. After briefly washing in TTBS, it was incubated with 1:1000 anti-phosphor-CREB (pCREB) (Ser-133, polyclonal; Cell Signaling Technology Inc., Beverly, MA) in 5% bovine serum albumin/TTBS overnight at 4°C. After 3 × 15 min washes with TTBS, the membranes were incubated with 1:10,000 anti-rabbit IgG horseradish peroxidase (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) in 5% skim milk/TTBS for 1 h at RT. After 3 × 15 min washes with Tris-buffered saline containing 0.3% Tween 20, it was developed with the ECL kit (Amersham Biosciences, Inc.) and digitized with LAS-1000 Plus Image Reader (Fuji Photo Film Co. Ltd., Tokyo, Japan). For detecting CREB, the membrane was stripped using Restore stripping buffer (Pierce Chemical, Rockford, IL) at RT for 15 min and washed 3 × 15 min with TTBS. After blocking with 5% skim milk/TTBS for 1 h, the membrane was incubated with 1:1000 polyclonal anti-CREB (Cell Signaling Technology Inc.) and detected using the same secondary antibody as described above.

Data Analysis. ^{125}I efflux rate was calculated following $[\ln(R_2) - \ln(R_1)]/(t_2 - t_1)$, where R_x is the percentage of radioactivity remaining in the monolayer at time t_x as described previously (Rogers et al., 1990). Data are expressed as mean ± S.E. of three or more independent experiments unless otherwise specified. Dose-response curves and IC_{50} values were analyzed by a nonlinear iterative regression routine with Grafit (Erithacus Software, Horley, Surrey, UK).

Results

PDE3 and PDE4 Are the Major cAMP-PDEs in T84 Cells. Human colonic T84 cells express abundant CFTR, AC, PKA, and multiple cAMP-PDEs as in human airway epithelial cells (Cohn et al., 1992; Sun et al., 2000; O'Grady et al., 2002). Previous studies also established that its cAMP-mediated chloride secretion, sensitive to the overexpression of a mutant PKA, occurred primarily through CFTR channel (Rogers et al., 1990; Bell and Quinton, 1992). In addition, T84 monolayer possesses several key characteristics that are similar to native secretory epithelial cells, including the formation of tight junctions and the maintenance of a vectorial chloride transport in the confluent stage. Thus, it provides the advantage of a nonrecombinant system to study CFTR regulation in response to AC activation and PDE inhibition (Halm et al., 1988).

To dissect the expression of cAMP-PDEs in T84 cells, the hydrolysis of cAMP by T84 lysate was titrated with the PDE4 inhibitor L-826,141 (Cpd-A), roflumilast, and the PDE3 inhibitor trequinsin. As summarized by their intrinsic potencies against the multiple recombinant cAMP-PDEs in Table 1, Cpd-A and roflumilast are potent PDE4 inhibitors, with IC_{50} values ranging from 0.2 to 2.4 nM and from 0.1 to 0.6 nM for the inhibition of PDE4A, 4B, 4C, and 4D, respectively. Cpd-A and roflumilast are >400- and 10,000-fold more potent than their weaker PDE3 inhibition, respectively (Claveau et al., 2004). Trequinsin, with IC_{50} value of ~0.05 nM against PDE3A and 3B, is >6000-fold more potent compared with its weaker PDE4 inhibition. The inhibition of the cAMP-PDE activity of T84 lysate by Cpd-A and roflumilast were biphasic (Fig. 1A). Each inhibited ~55% of the total activity

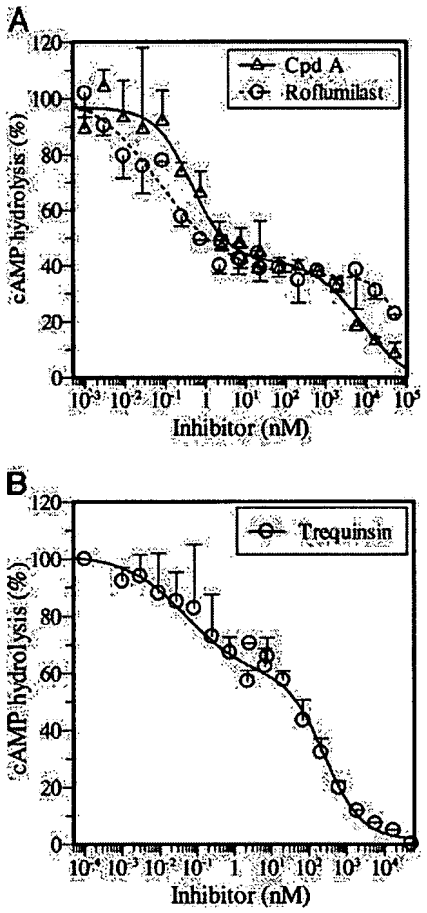


Fig. 1. A, inhibition of T84 lysate-catalyzed cAMP hydrolysis by roflumilast (○) and Cpd-A (△). The biphasic curves have the first inflection points at ~0.1 nM for roflumilast and ~0.4 nM for Cpd-A, with an overlapping plateau at 40 to 45% of the total activity. Cpd-A has a second inflection point at ~3000 nM. Roflumilast starts to inhibit the remaining activity above 5000 nM. Mean ± S.E. (n = 3). B, inhibition of T84 lysate-catalyzed cAMP hydrolysis by trequinsin. The biphasic curve has its first inflection point at ~0.1 nM with a plateau near 65% of the total activity. The second response has an inflection point near 400 nM. Mean ± S.E. (n = 3).

during the first titration phase, with inflection points at ~0.4 and ~0.1 nM, respectively. These values were consistent with their intrinsic potencies against the recombinant PDE4s listed in Table 1. Approximately 30% of the total activity was further inhibited by higher concentrations of Cpd-A with a second inflection point near 3000 nM, which is likely the consequence of its weaker PDE3 inhibition. This is supported by the biphasic titration of trequinsin from its potent PDE3 inhibition and weaker PDE4 inhibition in Fig. 1B. Its first and second titration phases each eliminated ~30 and ~55% of the total activity with the corresponding inflection points at ~0.1 and ~400 nM, respectively. Thus, PDE4 and PDE3 accounted for approximately 55 and 30% of the cAMP-PDE activity, respectively, in T84 lysate under the assay conditions. Most of the remaining activity (10 to 15% of the total) was inhibited by 10 μM 3-isobutyl-1-methylxanthine, a nonselective PDE inhibitor. This was thought to be contributed by PDE1 from its sensitivity toward 8-methoxymethyl-3-isobutyl-1-methylxanthine (O'Grady et al., 2002). The abundant PDE4 and PDE3 expression in T84 cells compared well with that in airway epithelial cells.

Forskolin-Stimulated Dynamic ^{125}I Efflux in T84 Cells. To monitor chloride secretion in T84 cells, we selected the well established $^{125}I^-$ efflux assay for its sensitivity and robustness. Previous head to head studies comparing $^{125}I^-$ and $^{36}Cl^-$ have established that $^{125}I^-$ is an ideal alternative for the highly penetrating $^{36}Cl^-$ in monitoring chloride secretion from T84 cells (Venglarik et al., 1990). After removing extracellular $^{125}I^-$ through extensive buffer washing, the efflux of intracellular $^{125}I^-$ into the incubation media was continuously monitored every 30 s by replacing *only* 80% of the incubation buffer with fresh buffer over a 9-min period. The partial buffer replacement minimized physical perturbation to the monolayer from repeated buffer exchange, thus significantly improved data reproducibility over that of previous procedures. Activators were added via the efflux buffer at 1.5 min, with their concentrations maintained throughout the remaining time course. More than 85% of the intracellular $^{125}I^-$ was released into the culture media over the 9-min duration in response to a saturating concentration (10 μM) of forskolin stimulation (Fig. 2A, ○). More than 90% of the released radioactivity comigrated with authentic $^{125}I^-$ by high-performance liquid chromatography analysis, indicating a negligible biotransformation of the tracer. Compared with the spontaneous efflux (Fig. 2B, (▼), significantly ele-

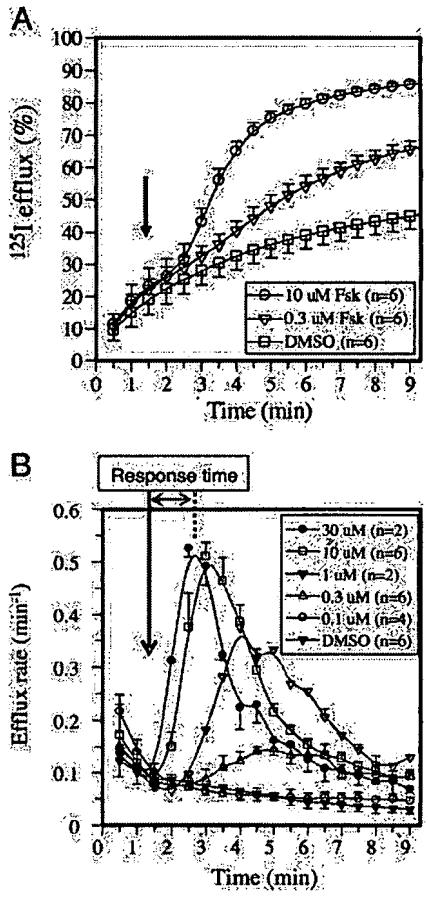


Fig. 2. A, time course of forskolin-induced ^{125}I efflux (percentage of total) from T84 monolayer at 37°C. Mean ± S.E. (n = 6). Forskolin was added at 1.5 min as marked by the arrow, and its concentration was maintained throughout the remaining duration. Forskolin (10 μM) (○), 0.3 μM forskolin (△), and vehicle (DMSO) (□) overlapped with that in the absence of DMSO. B, time course of increased ^{125}I efflux in response to increased forskolin stimulation. The efflux rate (minute^{-1}) was calculated from the efflux curves in A as detailed under *Materials and Methods*. The latency to reach the peak efflux rate after forskolin stimulation is defined as the response time as illustrated.

vated $^{125}I^-$ efflux slowly emerged after >5.5 min incubation with 0.1 μM forskolin (Fig. 2B, Fsk, ○). Higher forskolin concentrations elicited a faster and transient increase in ^{125}I efflux in a dose-dependent manner that remained elevated at approximately 2 to 3 times that of the spontaneous level at the end of the 9-min period. The peak efflux rate extrapolated from the efflux curves saturated in response to increasing forskolin concentrations with an EC_{50} of ~0.55 μM. In addition, the increased forskolin concentration progressively reduced the latency to reach the maximum efflux response (response time) as marked in Fig. 2B. These results are consistent with the presence of a tightly coupled AC-cAMP-CFTR signaling in T84 cells, reflecting a progressively activated CFTR from increased AC activation by forskolin. The iodide secretion peaked within 60 s after 30 μM forskolin stimulation, illustrating the rapid responsiveness of the system. The efflux rate peaked and then fell rapidly above 1 μM forskolin. The later phase of the biphasic response that causes the efflux rate to decline could be contributed either by tracer depletion, local ATP depletion, or desensitization. The forskolin-stimulated iodide efflux and its responsiveness in T84 cells echoed that in CFTR-overexpressing cells (Haws et al., 1996).

Synergistic CFTR Activation from Dual PDE3 and PDE4 Blockade under the Basal AC State. In the absence of forskolin, the iodide efflux was insensitive to the presence of up to 10 μM Cpd-A or 1 μM trequinsin, with their efflux curves overlapped with the spontaneous efflux of the cell (Fig. 3, □). Since these concentrations were >1000-fold above their intrinsic PDE4 and PDE3 potencies, respectively, a complete suppression of PDE4 and PDE3 activity would be expected under the conditions, even after considering the potentially reduced potency from increased protein binding in the whole cell environment. Yet, the ablation of PDE3 or PDE4 activity alone, under the basal AC state, was insufficient to activate the iodide secretion. On the other hand, dual PDE4 and PDE3 blockade (10 μM Cpd-A plus 1 μM trequinsin; Treq) induced a significantly elevated iodide secretion (Fig. 3, ▲), yielding an efflux curve that has a comparable peak efflux rate but with a prolonged duration to that elicited by 0.3 μM forskolin alone (Fig. 3, ▽). These data suggest that dual suppression of PDE3 and PDE4 activity is synergistic, instead of additive, in stimulating the cAMP-mediated CFTR activation under the basal AC state with a limited cAMP turnover.

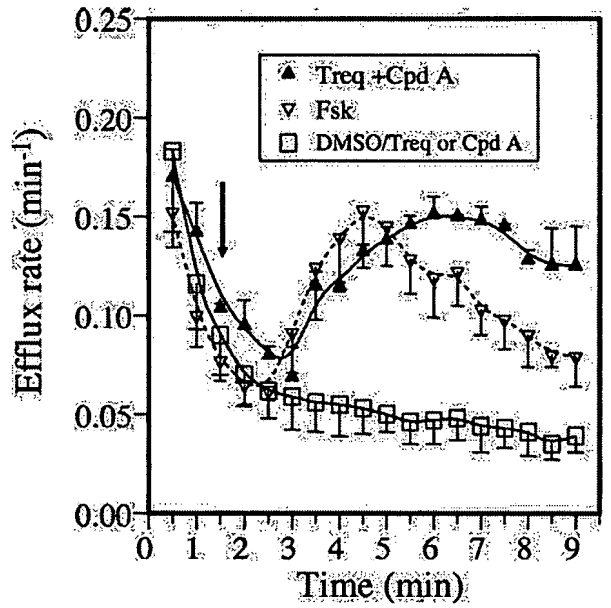


Fig. 3. PDE3, PDE4, and dual PDE3 and PDE4 inhibition elicited ^{125}I efflux in the absence of forskolin stimulation. Compounds were added at 1.5 min as marked, with their concentrations maintained throughout the remaining duration. The efflux curves of 10 μM Cpd-A or 1 μM trequinsin overlapped with the spontaneous efflux of DMSO control (□, DMSO). Cpd-A (10 μM) plus 1 μM trequinsin (▲) elicited an efflux response with a comparable peak efflux rate but a prolonged efflux duration in comparison with that induced by 0.3 μM forskolin (▽). Mean ± S.E. (n = 2–4).

Increased Efficacy of PDE3 and PDE4 Inhibitors upon Adenylyl Cyclase Activation. In contrast to the lack of efficacy under the basal AC state, PDE3 or PDE4 inhibition each potently augmented iodide secretion after forskolin stimulation. The presence of 0.1 μM forskolin, which only slightly increased the efflux after >5.5 min incubation (Fig. 2B, ○), significantly increased the efficacy of 1 μM Cpd-A by elevating the efflux rate to 0.055 min^{-1} over the vehicle control, with the enhanced efflux emerged after ~3 min instead. Under the same AC state, 0.1 and 1 μM trequinsin augmented the peak efflux to 0.09 and 0.16 min^{-1}, respectively. Further AC activation by increasing forskolin to 0.3 μM amplified the responsiveness of the iodide secretion toward PDE4 and PDE3 inhibition, as represented by the efflux curves of Cpd-A (Fig. 4A) and trequinsin (Fig. 4B). Cpd-A (3 nM) (Fig. 4A, ▼) or 1 nM trequinsin (Fig. 4B, ▼) each significantly prolonged the iodide efflux induced by 0.3 μM forskolin (Fig. 4, A and B, ○), and 3 nM roflumilast elicited a similar response to that of Cpd-A (data not shown). Higher inhibitor concentrations augmented the peak efflux rate in a dose-dependent and biphasic manner, with the response of Cpd-A nearly superimposed onto that of roflumilast (Fig. 4C). The first phases of Cpd-A and roflumilast's biphasic responses elevated the peak efflux rate by ~0.06 min^{-1} over the forskolin response, with inflection points between 2 to 5 nM. Their second response phases started above 1000 nM, increasing the peak efflux rate by another ~0.09 min^{-1}. In comparison, trequinsin augmented the peak efflux rate by ~0.09 min^{-1} over the forskolin response with an initial inflection point between 1 to 3 nM. Its second response phase occurred above 100 nM, elevating the peak efflux rate by another ~0.05 min^{-1}, which is comparable with that derived from PDE4 inhibition by Cpd-A or roflumilast. The potentiating effects through PDE3 or PDE4 inhibition start to diminish at higher AC activation states, with a minimal augmentation detected at or above 10 μM forskolin (data not shown).

The increased PDE3 or PDE4 inhibition also progressively reduced the response time. This became more apparent near their saturating doses, particularly judging from the faster rising phase of their efflux curves in Fig. 4, A and B. At the submaximally activated AC state by 0.3 μM forskolin, the ablation of PDE4 and PDE3 activity by 1 μM Cpd-A plus 1 μM trequinsin elicited a peak efflux response (Fig. 5, ○) which was comparable with that from a full AC activation by 10 μM forskolin (Fig. 5, △). The combination of higher PDE3 and PDE4 inhibitor concentrations did not augment the peak efflux rate nor shorten the response time further, supporting that PDE3 and PDE4 are mainly responsible for degrading the cAMP pool involved in CFTR activation in T84 cells.

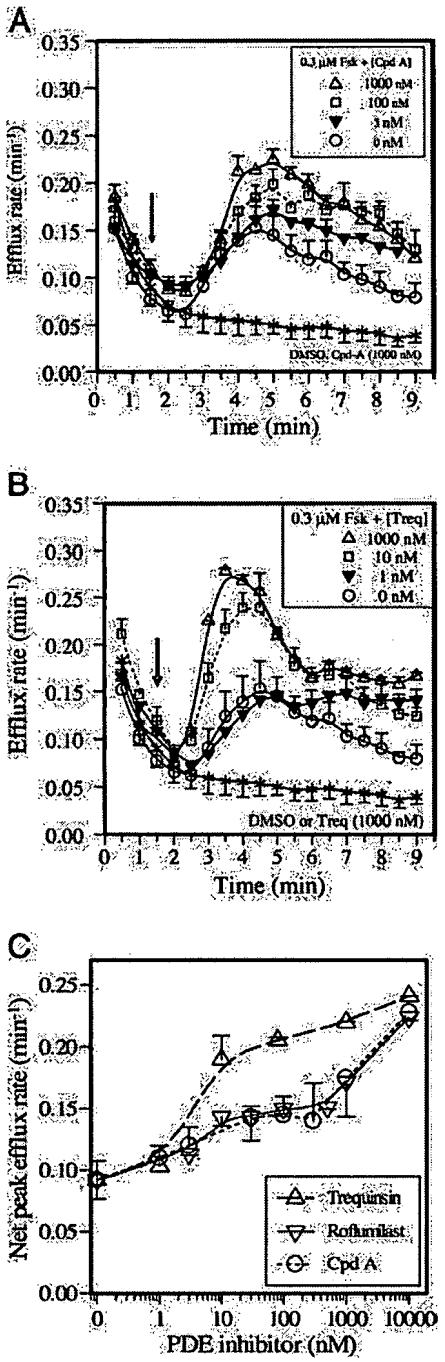


Fig. 4. A, time course of Cpd-A elicited ^{125}I efflux in the presence of 0.3 μM forskolin. Increased concentrations of Cpd-A and 0.3 μM forskolin were added simultaneously at 1.5 min as marked by the arrow, with their concentrations maintained throughout the remaining duration. DMSO control (*), 0.3 μM forskolin (○), 0.3 μM forskolin plus 3 nM Cpd-A (▼), 0.3 μM forskolin plus 100 nM Cpd-A (□), 0.3 μM forskolin plus 1000 nM Cpd-A (△). Mean ± S.E. (n = 2–4). B, time course of trequinsin elicited ^{125}I efflux in the presence of 0.3 μM forskolin. Increased concentrations of trequinsin and 0.3 μM forskolin were added simultaneously at 1.5 min as marked by the arrow, with their concentrations maintained throughout the remaining duration. DMSO control (*), 0.3 μM forskolin (○), 0.3 μM forskolin plus 1 nM trequinsin (▼), 0.3 μM forskolin plus 10 nM trequinsin (□), 0.3 μM forskolin plus 1000 nM trequinsin (△). Mean ± S.E. (n = 2–4). C, net peak efflux rate from PDE3 and PDE4 inhibition in the presence of 0.3 μM forskolin. The average net peak efflux rates over the DMSO control in response to increased PDE3 and PDE4 inhibition were summarized. Cpd-A (○), roflumilast (▽), and trequinsin (△). Data were from cells with similar passage numbers. Mean ± S.E. (n = 4–6).

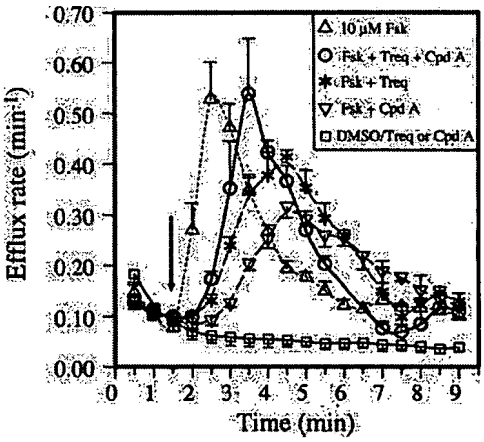


Fig. 5. Chloride efflux from maximal PDE3, PDE4, and PDE3 plus PDE4 inhibition in the presence of 0.3 μM forskolin compared with that from 10 μM forskolin. All compounds were added at 1.5 min as marked by the arrow, with their concentrations maintained throughout the remaining duration. DMSO control (□), 0.3 μM forskolin plus 1 μM Cpd-A (▽, peak efflux ~0.3 min^{-1}, response time ~180 s), 0.3 μM forskolin plus 1 μM trequinsin (*, peak efflux ~0.4 min^{-1}, response time 150–180 s), 0.3 μM forskolin plus 1 μM trequinsin and 1 μM Cpd-A (○, peak efflux ~0.53 min^{-1}, response time ~120 s), and 10 μM forskolin (△, peak efflux ~0.52 min^{-1}, response time ~60 s). The same batch of cells was used. Mean ± S.E. (n = 2–4).

Compared with forskolin stimulation, there remains a delayed response in activating the chloride secretion from blocking cAMP degradation through PDE3 plus PDE4 inhibition. The exact timing difference remains to be quantified with a faster sampling protocol.

CFTR Activation, Paralleled with Phosphor-CREB Formation, Is Blocked by H89. pCREB induction is a surrogate for PKA activation from cAMP elevation. The CFTR activation through PDE4 inhibition was compared with increased pCREB formation under the same condition. Treatment of T84 cells with 1 μM Cpd-A alone caused no pCREB induction, echoing its negligible stimulation of iodide secretion. In contrast to the robust activation of iodide secretion after 2 min, 0.3 μM forskolin nonsignificantly elevated the pCREB level over the DMSO control after 10 min (1.6-fold; $p < 0.2$; Fig. 6). Further cAMP elevation by combining 0.3 μM forskolin and 1 μM Cpd-A resulted in a statistically significant pCREB induction over the DMSO control (1.8-fold at 5 min, $p < 0.02$; 3-fold at 10 min, $p < 0.001$; Fig. 6). Thus, pCREB induction is a less sensitive marker for PKA activation in comparison with the active iodide secretion.

To confirm the increased iodide secretion from PDE4 inhibition was through PKA activation, cells were pretreated with the PKA inhibitor H89 (10 μM) for 10 min before Cpd-A and forskolin stimulation, with H89 concentration maintained throughout the remaining duration. Approximately 55% of the efflux induced by 1 μM Cpd-A plus 0.3 μM forskolin was blocked by H89 treatment, judging from the reduced area under the curves over the DMSO control (Fig. 7A). The H89-suppressed efflux was associated with ~66% reduced pCREB formation (Fig. 7B).

Stereoselective Activation of the Iodide Efflux by a PDE4D-Selective Inhibitor. To delineate the PDE4 subtype involved, we compared the efficacy of 2-(4-fluorophenoxy)-*N*-[(1*S*)-1-(4-methoxyphenyl)ethyl]nicotinamide (Cpd-B) with its (*R*)-isomer (Cpd-C) in augmenting the iodide efflux in the presence of 0.3 μM forskolin. This pair of enan-

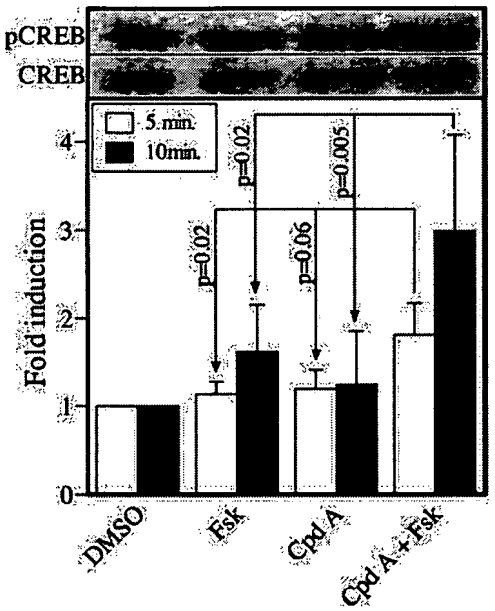


Fig. 6. Phosphor-CREB induction after Cpd-A and forskolin stimulation. pCREB levels after 5- and 10-min treatment with 0.3 μM forskolin, 1 μM Cpd-A, and 0.3 μM forskolin plus 1 μM Cpd-A were analyzed by Western blot analysis. Top, pCREB and CREB blot images after drug treatment for 5 min; the CREB levels were unchanged among all samples. Image intensities of pCREB levels over the DMSO control were plotted in the bar graph. Mean ± S.E. (n = 4). DMSO treatment had no effect on pCREB level. Its induction by 0.3 μM forskolin (1.6-fold after 10 min; $p < 0.2$) and by Cpd-A were statistically insignificant. The combination of Cpd-A plus forskolin significantly elevated pCREB formation by 1.8-fold after 5 min ($p < 0.02$), which increased to 3-fold after 10 min ($p < 0.001$). These values were also statistically higher than the pCREB levels after forskolin or Cpd-A treatment alone as indicated by the p values on the graph.

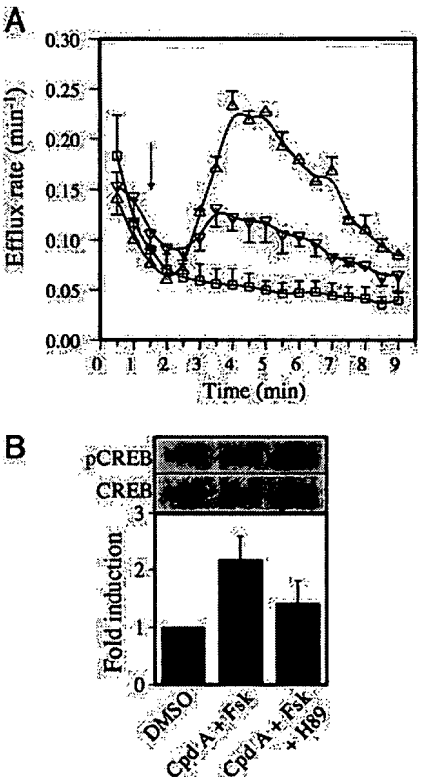


Fig. 7. A, H89 inhibition of ^{125}I efflux induced by Cpd-A plus forskolin. H89 (10 μM) was added 10 min before the efflux initiation, and its concentration was maintained throughout the remaining time course. Cpd-A (1 μM) plus 0.3 μM forskolin was added at 1.5 min simultaneously as marked by the arrow to initiate the active ^{125}I secretion. Cpd-A (1 μM) plus 0.3 μM forskolin (△), 10 μM H89 plus 1 μM Cpd-A plus 0.3 μM forskolin (▽), and vehicle DMSO (□). H89 suppressed the ^{125}I efflux by ~55% from the reduced area under the curve over the DMSO control. B, corresponding pCREB levels after 10-min drug treatment as in A. Forkolin (Fsk, 0.3 μM) plus 1 μM Cpd-A increased pCREB level by 2.2 ± 0.4-fold ($p < 0.04$) over the DMSO control. Pretreatment with 10 μM H89 reduced the pCREB induction by ~66%. Mean ± S.E. (n = 2).

tiomers was discovered by scientists at Pfizer, Inc. (Marfat and Chambers, 1998). The (*S*)-isomer stereoselectively inhibited PDE4D with an IC_{50} of 1.4 nM under our assay conditions (Table 1). It is >140-fold more potent against PDE4D in comparison with its inhibition of PDE4A, 4B, and 4C. In addition, the (*S*)-isomer is an ~28-fold more potent PDE4D inhibitor than the less potent and less selective (*R*)-isomer. As shown in Fig. 8, significantly augmented iodide secretion over the forskolin-control was detected at 1 nM ($p < 0.05$; n = 4) and 5 nM ($p < 0.001$; n = 4) of the (*S*)-isomer, which are comparable with that elicited by the nonselective Cpd-A or roflumilast within a similar concentration range. The efficacy of the (*R*)-isomer decreased in parallel by approximately 10- to 20-fold at augmenting the peak efflux rate. Thus, the efficacy difference of the two stereoisomers closely matched their PDE4D potency difference, supporting that PDE4D is the predominant PDE4 component in regulating the active iodide secretion in T84 cells.

CFTR Activation Dissociated from Global Intracellular cAMP Elevation. The cAMP content of T84 cells in response to forskolin stimulation and PDE4 or PDE3 inhibition was analyzed as detailed under *Materials and Methods*. Significantly elevated cAMP was only detected after 10-min treatment with >1 μM forskolin (Fig. 9). In the absence of forskolin, treatment with 1 μM Cpd-A, roflumilast, and trequinsin separately or their combination elicited a negligible global cAMP elevation. In the presence of 0.3 μM forskolin, elevated cAMP was only detected after treatment with above 5 μM trequinsin [FSK*/Treq(5)] or roflumilast [FSK*/Rof (5)], which are >1000-fold higher than the minimal dose capable of prolonging the efflux duration. Therefore, the global cAMP elevation from either forskolin stimulation or in combination with PDE3 or PDE4 inhibition all dissociated from their more potent activation of iodide secretion. It is also less sensitive than the pCREB induction index.

Discussion

The present results demonstrate that PDE3, PDE4, and PDE4D inhibitors each dynamically augment the CFTR-mediated iodide secretion in T84 cells after forskolin stimulation, with their efficacy coupled to the forskolin concentration. The enhanced iodide secretion in response to increased PDE4 or PDE3 inhibition was characterized initially by a prolonged efflux duration, followed by a progressively increased efflux rate and reduced response time at higher inhibitor concentrations. The peak iodide efflux from PDE4 plus PDE3 blockade matched the maximal response from a full AC-activation, with approximately 33% of the total efflux rate attained by PDE4 inhibition and the remaining by PDE3 inhibition. The increased iodide secretion from forskolin stimulation and PDE4 inhibition was associated with an increased pCREB formation at higher activation states and dissociated from a global cAMP elevation. H89 antagonized

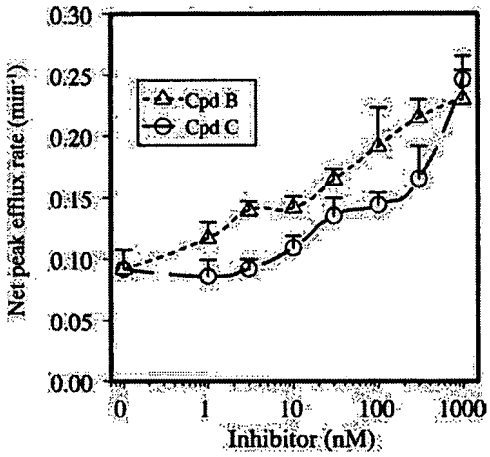


Fig. 8. Stereoselective augmentation of the peak efflux rate in response to increased PDE4 inhibition by Cpd-B and Cpd-C. The net peak efflux rates (over DMSO control) were determined in the presence of 0.3 μM forskolin. The PDE4D-selective (*S*)-isomer (Cpd-B, △) stereoselectively augmented the chloride efflux over the less potent and less selective (*R*)-isomer (Cpd-C, ○) in T84 cells. Mean ± S.E. (n = 3–4).

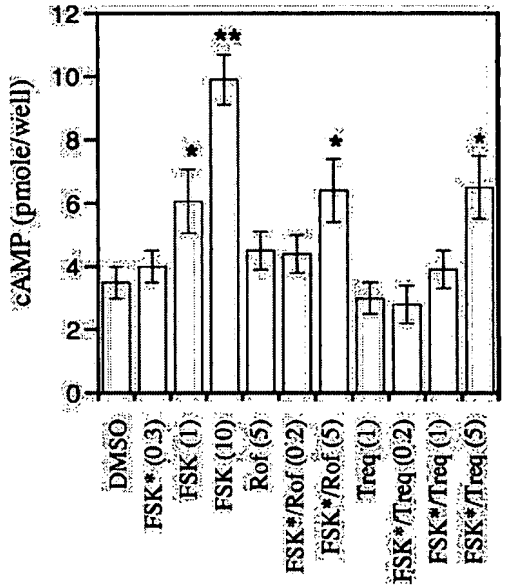


Fig. 9. Intracellular cAMP content of T84 cells after drug treatment for 10 min. The cAMP levels after forskolin treatment for 3 and 6 min were similar to those shown after 100-min treatment. FSK (x), Rof (x), and Treq (x) represent post-treatment with x μM forskolin, roflumilast, and trequinsin, respectively. FSK*/Rof (x) and FSK*/Treq (x) represent post-treatment with 0.3 μM forskolin plus x μM roflumilast or trequinsin, respectively; *, $p < 0.05$; **, $p < 0.001$. Mean ± S.E. (n = 6).

the forskolin-stimulated and PDE4 inhibitor-potentiated iodide secretion and pCREB formation in parallel. A possible explanation for these data is 1) the presence of a highly compartmentalized cAMP-mediated CFTR activation through PKA activation in T84 cells; and 2) PDE3 and PDE4, mainly through PDE4D, are responsible for ensuring the compartmentalized signaling by restricting cAMP diffusion through degradation.

The localized cAMP-CFTR signaling relies on the formation of a supermolecular assembly for specificity and efficiency. Previous studies have elegantly demonstrated the presence of a cAMP microdomain near its production site, with restricted diffusion access of cAMP to the bulky cytosol. PDE inhibitors reduced the compartmentalized response to various degrees (Rich et al., 2001; Jurevicius et al., 2003). A macromolecular complex including β_2-adrenoceptor, PKA, and CFTR through the interaction of protein kinase A anchoring proteins has been identified after receptor stimulation (Sun et al., 2000; Naren et al., 2003). Augmentation of the adenosine-induced apical anion conductance by RS25344 supports the PDE4 proximity to the signaling complex (Barnes et al., 2005). Stimulation of the β_2-adrenoceptor also rapidly recruits β-arrestins with bound PDE4s to the plasma membrane within minutes as part of the desensitization machinery to limit the spread of the cAMP pool (Baillie et al., 2003). Some PDE4s are associated with PKA via protein kinase A anchoring proteins and/or activated by PKA-mediated phosphorylations, which provide additional controls to ensure a localized cAMP signaling through PDE4 regulation (Laliberte et al., 2002; Conti et al., 2003). Despite its lower abundance in T84 cells, PDE3 inhibition by trequinsin seems to be a more effective activator of iodide efflux with a quicker response, compared with that from PDE4 inhibition by Cpd-A under an identical AC state. It is unclear whether the enhanced PDE3/CFTR coupling is due to their potential proximity or from the approximately 10-fold enhanced cAMP affinity of PDE3. Previous subfractionation study indicated a similarly abundant PDE4 over PDE3 distribution on the T84 membrane (O'Grady et al., 2002). Whether PDE3, PDE4, or PDE4D is physically associated with the CFTR regulatory complex remains to be clarified.

Airway disease from mucus accumulation, recurring bacterial colonization, and chronic inflammation is the major cause of morbidity and mortality in CF. Asthma, COPD, and chronic bronchitis are also characterized by chronic airway inflammation, mucus-congested airways, and hyperplasia of goblet cells. The shifted balance from water secretion to mucus secretion around the periciliary layer of the disease airways may compromise the mucociliary clearance process, with the resulting accumulation of mucus, bacterial, and host-response products from infiltrating neutrophils and eosinophils contributing significantly to the pathogenesis of disease states (O'Byrne and Postma, 1999). In contrast to CF, most asthma, COPD, and chronic bronchitis patients have functional CFTR channel. Agents such as PDE4 and PDE4D inhibitors that can reduce the exuberant inflammation response and simultaneously enhance mucociliary clearance through CFTR activation in airway may provide additional benefits over antiinflammatory treatment alone in their management. Cpd-A and roflumilast belong to the second generation nonselective PDE4 inhibitors, effectively suppressing the overproduction of leukotrienes and a variety of proinflammatory cytokines in vitro and in animal models (Hatzelmann and Schudt, 2001; Claveau et al., 2004). Treatment with roflumilast at 0.5 mg once daily has significantly improved airway function in asthmatic and COPD patients, with its plasma concentration reached a C_{max} of 3.8 ng/ml (~9.5 nM) and the active *N*-oxide metabolite being several-fold higher (Reid, 2002). Since the combined exposure is higher above the minimal dose required for CFTR activation in T84 cells, it is possible that an enhanced airway mucociliary clearance from CFTR activation may have contributed to its improved airway function in clinic. Slightly increased diarrhea incidence has been noted as a treatment-related adverse event in roflumilast trials with its mechanism(s) unresolved. Whether the enhanced secretary response signi-

fied a possible CFTR activation in the gastrointestinal track remains to be clarified. The present data clearly demonstrated that the cellular efficacy of PDE inhibitors is coupled to the AC activation state. If the flux-mediated sensitivity phenomenon is transferred in vivo, it would support the notion that PDE4 inhibitors are more effective at targeting disorders and potentiating pathways undergoing higher cAMP turnover. In view of the high flux sensitivity of PDE4 inhibitors in activating CFTR identified here, one might expect that the combination of an oral PDE4 inhibitor with an inhaled β-agonist may augment their efficacy and improve the therapeutic index further from the airway-specific activation of cAMP signaling.

In summary, the cAMP-mediated CFTR activation in T84 cells is almost exclusively under PDE3 and PDE4 (mainly PDE4D) regulation. The similarly abundant PDE4 and PDE3 expression in human airway epithelia supports an analogous CFTR regulation there. In view of the increased cardiotonic risk from the chronic administration of PDE3 inhibitors, restoring or enhancing the airway mucociliary clearance and suppressing the inflammatory responses with the emerging PDE4 inhibitors may provide exciting new opportunities to combat cystic fibrosis and other airway diseases, including asthma, COPD, and chronic bronchitis in near future.

Acknowledgments

We thank Merv Turner, Joseph Mancini, Brian Kennedy, Gary O'Neill, Dwight Macdonald, Yves Girard, Don Nicholson, and Robert Young (Merck Frosst) and Professor John Hanrahan (McGill University, Montreal, QC, Canada) for many helpful discussions.

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